

**INVESTIGATION OF THE POLYPYRIMIDINE TRACT-BINDING PROTEIN-ASSOCIATED
SPLICING FACTOR (PSF) DOMAINS REQUIRED FOR THE HEPATITIS *DELTA* VIRUS (HDV)
REPLICATION**

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Abstract

The hepatitis *delta* virus (HDV), composed of ~1,700nt, is the smallest circular RNA pathogen known to infect humans. Understanding the mode of replication of HDV implies on investigating the host proteins that bind to its genome. The polypyrimidine tract-binding protein-associated splicing factor (PSF), an HDV interacting protein, was found to interact with the carboxy terminal domain (CTD) of RNA polymerase II (RNAPII), and to facilitate the interaction of RNA transcripts with the CTD of RNAPII. Both PSF and RNAPII were found to interact with both polarities of the terminal stem loop domains of HDV RNA, which possess RNA promoter activity *in vitro*. Furthermore, PSF and RNAPII were found to simultaneously interact with HDV RNA *in vitro*. Together, the above experiments suggest that PSF acts as a transcription factor during HDV RNA replication by interacting with both the CTD of RNAPII and HDV RNA simultaneously. PSF knockdown experiments were performed to indicate that PSF is required for HDV RNA accumulation. Mutagenesis experiments of PSF revealed that HDV RNA accumulation might require the N terminal domain, and the RNA recognition motifs RRM1 and RRM2. I propose that the RRM1 and RRM2 domains might interact with HDV RNA, while the N-terminal domain might interact with the CTD of RNAPII for HDV RNA accumulation. Together, the above experiments provide a better understanding of how an RNA promoter might be recognized by RNAPII.

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List Of Abbreviations

- ADAR- Adenosine deaminase acting on RNA
- ARM- Arginine-rich-motif
- ASF- Alternative splicing factor
- bp- base pairs
- cDNA- complimentary deoxyribonucleic acid
- CTF/NF- 1- CCAAT- binding transcription factor/nuclear factor 1
- CTD- Carboxy terminal domain
- DNA- deoxyribonucleic acid
- DRB- 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole
- dsDNA- double stranded deoxyribonucleic acid
- DSIF- DRB-sensitivity inducing factor
- dsRNA- double stranded ribonucleic acid
- eEF1A1- Eukaryotic translation elongation factor 1A1
- EMSA- Electrophoretic mobility shift assay
- GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
- GST- glutathione S transferase
- GTF- General transcription factor
- HBV- Hepatitis B virus
- HDV- hepatitis *delta* virus
- HIV-1- human immunodeficiency virus type 1
- HLH- helix loop helix
- hnRNPL- heterogeneous nuclear ribonucleoprotein L
- K_D - Dissociation constant

kDa- Kilodalton

mRNA- messenger ribonucleic acid

NE- Nuclear extract

NELF- negative elongation factor

NES- nuclear export signal

NLS- Nuclear localization signal

nt- nucleotide

NTP- Nucleoside triphosphate

ORF- open reading frame

p54^{nrb}- 54kDa nuclear RNA-binding protein

PAGE- polyacrylamide gel electrophoresis

PCR- polymerase chain reaction

PIC- pre-initiation complex

PKR- double stranded RNA activated protein kinase R

PLMVd- peach latent mosaic viroid

PRRSV- porcine reproductive and respiratory syndrome virus

PTB- Polypyrimidine tract binding protein

P-TEFb- Positive transcription elongation factor B

PSF- Polypyrimidine tract binding protein associated splicing factor

qPCR- Quantitative polymerase chain reaction

RACE- rapid amplification of cDNA ends

RBD- RNA binding protein

RIPA- Ribonucleoprotein immunoprecipitation assay

RISC- RNA-induced silencing complex

RNA- ribonucleic acid

RNAP- RNA polymerase

RNAPI- RNA polymerase I

RNAPII- RNA polymerase II

RNAPIII- RNA polymerase III

RRM- RNA recognition motif

RNP- Ribonucleoprotein

RT- PCR- Reverse transcriptase-polymerase chain reaction

δ ag- S- Hepatitis *delta* antigen (small isoform)

δ ag- L- Hepatitis *delta* antigen (large isoform)

SC35- 35 kDa splicing factor

SELEX- systematic evolution of ligands by exponential enrichment

SFPQ- Splicing factor proline/glutamine-rich

siRNA- small interfering ribonucleic acid

SRCR- cysteine rich domain 5

TET- tetracycline

TFIIS- RNA polymerase II transcription factor IIS

(u)- Units

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1.0. Introduction

1.1. Hepatitis *delta* virus (HDV) infection and pathogenesis

Approximately 400 million people are chronically infected with hepatitis B virus (HBV) and it is estimated that 10% are co-infected with hepatitis *delta* virus (HDV) (reviewed in Taylor, 2003; Taylor, 2006). HDV was discovered in 1977 as a foreign antigen from the serum and the liver of Italian individuals chronically infected with HBV (Rizzetto *et al.*, 1980). HDV, composed of ~1,700nt, is the smallest circular RNA pathogen known to infect humans (Gudima *et al.*, 2007a). An HDV infection requires HBV as a helper virus making it a satellite virus (Gudima *et al.*, 2007a). Because HDV utilizes the HBV surface antigens for its encapsidation and transmission, natural HDV infection occurs only when co-infected with HBV or from a superinfection, which results when HBV carriers get co-infected with HDV (Macnaughton and Lai, 2006; Gudima *et al.*, 2007b). A co-infection with both viruses causes rapid liver damage due to fulminate acute and an end-stage chronic HDV infection, where liver transplants are required (reviewed in Hadziyannis, 1997, Ivaniushina *et al.*, 2001). Large doses of α -interferon can be administered to slightly improve symptoms caused by an HDV infection (Farci *et al.*, 1994; Gunsar, *et al.*, 2005). However, currently the vaccination against HBV is the key in preventing an individual from getting co-infected with both HBV and HDV, since without the HBV surface antigens, HDV propagation does not occur (reviewed in Hadziyannis, 1997, Ivaniushina *et al.*, 2001; reviewed in Sureau, 2006).

HBV contains three surface antigens; the large (L), medium (M) and small (S) which are utilized by HDV for its encapsidation and transmission (reviewed in Sureau, 2006). These three proteins have unique domains that allow them to function distinctively during an

infection. Relative to the S protein, the M protein has a unique N-terminal domain referred to as the preS2. Relative to the M, the L protein has a unique domain referred to as the preS1. These proteins undergo a series of posttranslational modifications leading to the release of infectious particles (Sureau *et al.*, 1994). The M protein is not required for the assembly or the infectivity of HBV or HDV (Sureau *et al.*, 1994), but the stoichiometry of the L to S proteins is critical for the infectivity of both viruses (reviewed in Sureau, 2006). The L protein is essential for both the assembly and the infectivity of both viruses (Sureau *et al.*, 1993) but in the absence of the S protein, the L is incapable of being released from the cell (reviewed in Sureau, 2006). On the other hand the S protein is adequate for the assembly of the particles but is noninfectious (Sureau *et al.*, 1993).

The ~1,700nt HDV genome contains a single open reading frame encoding two proteins: the small *delta* antigen δ ag-S (composed of 195 aa, 24kDa) and the large *delta* antigen (δ ag-L) (Figure 1.1) (composed of 214aa, 27kDa) (Casey and Gerin, 1995). The δ ag-S is required for HDV RNA replication (Casey and Gerin, 1995; reviewed in Lai, 1995), while the δ ag-L acts to inhibit replication but is essential for virus assembly (Chang *et al.*, 1991; Casey and Gerin, 1995; reviewed in Lai, 1995). Therefore, in addition to utilizing the three HBV surface antigens, HDV needs to form a ribonucleoprotein (RNP) complex, which consists of circular genomic HDV RNA and approximately 70 copies of the HDV *delta* antigens in order for an HDV infection to occur (Figure 1.2) (Bonino *et al.*, 1984; reviewed in Sureau, 2006). The exact process in which this ribonucleoprotein complex infects hepatocytes remains elusive, however it is hypothesized that it is mediated via the interaction of the preS1 to a hepatocyte surface receptor (reviewed in Taylor, 2003; Taylor, 2006).

Figure 1.1: The hepatitis *delta* virus (HDV). The hepatitis *delta* genome is depicted here with the superimposition of both genomic and antigenomic polarities. The HDV RNA genome is shown here as a single stranded RNA molecule that folds to form an unbranched rod-like structure. The two complimentary ribozyme motifs, cleavage sites, and the open reading frame (ORF) are indicated. R199G represents 199 nucleotides derived from the right terminal stem loop domain of the genomic polarity of HDV. R199G is reported to have promoter activity *in vitro* (Beard *et al.*, 1996; Gudima *et al.*, 1999; Abraham and Pelchat, 2008). Numbering is in accordance with Kuo *et al.*, 1988. (Figure adapted from Greco-Stewart *et al.*, 2007; Figure 1).

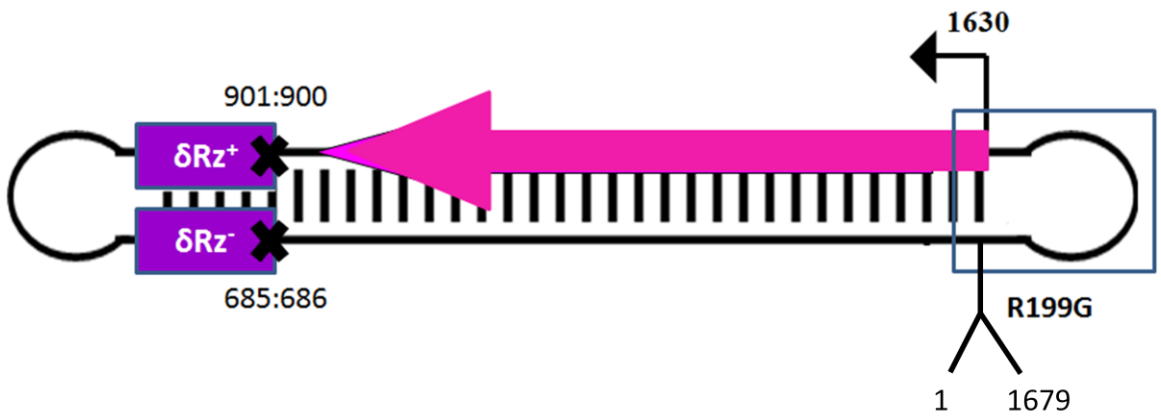
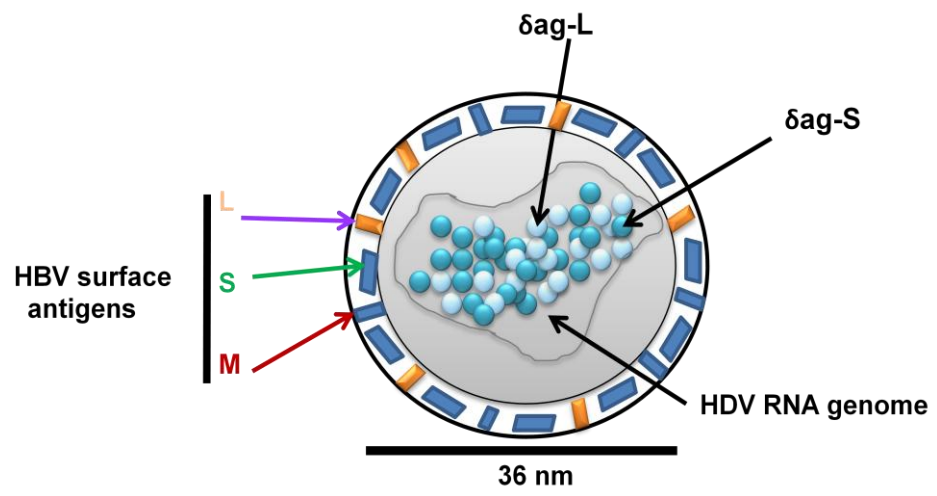


Figure 1.2: Schematic representation of a hepatitis *delta* virion particle. HDV RNA uses the three hepatitis B surface antigens; the small (S), medium (M), and large (L) to encapsidate itself and transmit inside cells. The δ ag-L and δ ag-S *delta* antigens are also required for infection and replication of HDV.



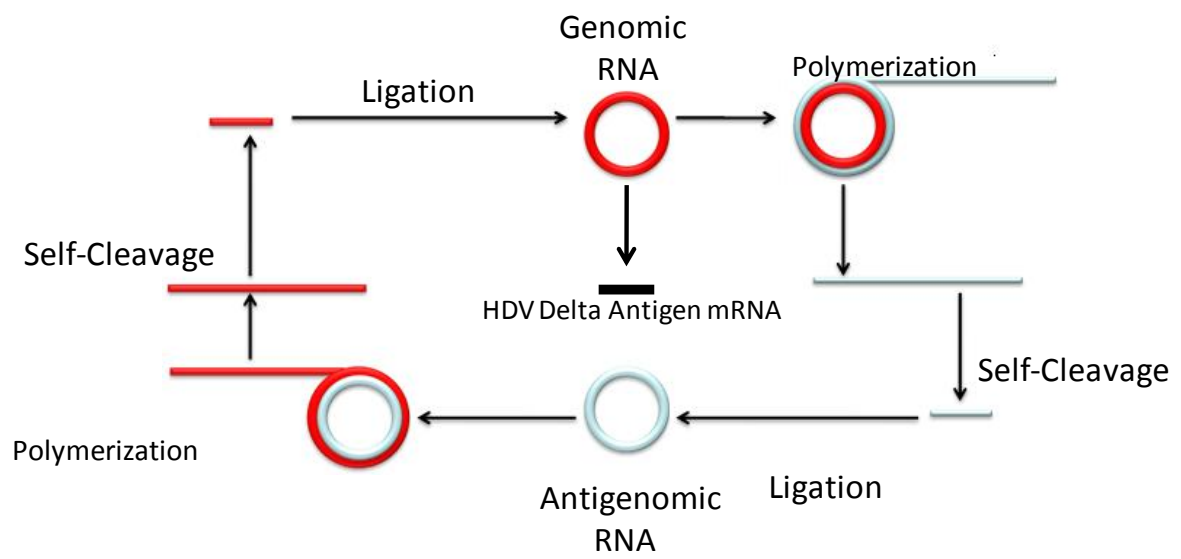
The HDV ribonucleoprotein is then uncoated from the HBV surface antigens and enters the nucleus. However, HDV RNA remains bound to the δ ag-S possibly because it aids its import into the nucleus, and protects it from degradation (Chang *et al.*, 1995).

1.2. HDV RNA replication

Although HDV relies on HBV for infection, it does not rely on HBV for its replication (reviewed in Beck and Nassal, 2007). Rather the replication of HDV differs from that of HBV. HBV replication involves reverse transcription and the accumulation of circular double stranded DNA species which are required for the transcription of new RNA species (reviewed in Beck and Nassal, 2007). HDV replication only generates RNA species without using DNA intermediates. The accepted mechanism for HDV RNA replication is the symmetrical double rolling circle mechanism (Figure 1.3). The symmetrical double rolling mechanism begins when an infectious monomeric circular genomic RNA strand is transcribed by a host RNA polymerase to generate linear multimeric antigenomic RNA strands. These linear multimeric antigenomic strands are then cleaved by endogenous *delta* ribozyme motifs into unit length monomeric antigenomic strands [the *delta* ribozyme motifs present on HDV RNA (nucleotides 659-772) *cleave* genomic multimeric species (at nucleotides 685-686-Figure 1.1). On the other hand distinct *delta* ribozyme motifs cleave antigenomic HDV RNA multimeric species (at nucleotides 901-900-Figure 1.1) into unit length monomeric species (Kuo *et al.*, 1988; Wu *et al.*, 1989)].

Figure 1.3: HDV replicates by the symmetrical double rolling circle mechanism.

Polymerization of the circular genomic monomeric strand occurs via a host RNA polymerase which produces linear antigenomic RNA multimers. The RNA multimers are then cleaved by *delta* ribozyme motifs into RNA monomers which are then ligated by host ligases to produce circular antigenomic RNA monomers. The antigenomic RNA monomers then serve as templates for genomic RNA transcripts. Genomic RNA transcripts also serve as templates to generate the mRNA for the HDV delta antigens. The antigenomic linear monomeric strands are then ligated by unknown host ligases to produce circular antigenomic monomeric strands. The circular antigenomic monomeric strands are then used as templates to generate linear genomic multimeric strands. The genomic multimeric strands are then cleaved and ligated into linear monomeric strands, which serve as templates to generate more RNA transcripts (reviewed in Taylor, 2006).



Thus, HDV RNA replication refers to the production of genomic and antigenomic RNA transcripts that are transcribed from antigenomic and genomic RNA templates, respectively. Both the antigenomic and genomic strands fold intramolecularly to form unbranched rod-like structures (Figure 1.1, Kuo *et al.*, 1988). Approximately 74% of the nucleotides in HDV RNA possess canonical intramolecular base pairing (Kuo *et al.*, 1988). In addition to the accumulation of the genomic and antigenomic strands during HDV RNA replication, a third RNA is produced from the genomic strand, the 800nt mRNA which encodes for either the δ_{ag-S} or the δ_{ag-L} .

1.3. HDV RNA transcription and replication is dependent on DNA-dependent RNA polymerases

For decades it has been widely accepted that prokaryotic and eukaryotic DNA-dependent RNA polymerases only transcribe from a DNA template. However, the discovery of infectious subviral RNA molecules, such as HDV RNA, changed the understanding of how DNA-dependent RNA polymerases select their templates. HDV replication requires δ_{ag-S} (Dingle *et al.*, 1998; Kuo *et al.*, 1989). However, δ_{ag-S} does not possess RNA polymerase activity. Therefore, how does this RNA transcribe from an RNA template in the absence of an RNA-dependent RNA polymerase? It has been proposed that HDV must rely on a host DNA-dependent RNA polymerase for its transcription and replication.

The δ_{ag-S} reverses HDV RNA transcription repression imposed by the negative elongation factor (NELF) (Yamaguchi *et al.*, 2001). NELF is a transcriptional regulatory factor that interacts with DNA-dependent RNA polymerase II (RNAP II) to inhibit transcription elongation (Yamaguchi *et al.*, 2001). Transcription is repressed by both NELF and the DRB

sensitivity inducing factor (DSIF). However, transcription elongation can proceed when the positive transcription elongation factor B (P-TEFb) phosphorylates both DSIF and the carboxy terminal domain (CTD) of RNAP II (Yamaguchi *et al.*, 2001). The Handa laboratory reported that both *delta* antigens bind RNAP II directly, and stimulate transcription by displacing NELF to promote transcription elongation by RNAPII (Yamaguchi *et al.*, 2001; reviewed in Greco-Stewart and Pelchat, 2010). The HDV *delta* antigens, and particularly the δ ag-S stimulates transcription elongation in the absence of DSIF/NELF, while it reverses transcription repression imposed by DSIF/NELF (Yamaguchi *et al.*, 2001; reviewed in Greco-Stewart and Pelchat, 2010). This was demonstrated by the addition of δ ag-S during *in vitro* transcription of HDV RNA (using HeLa nuclear extracts). HDV RNA transcription was repressed upon the addition of NELF. The repression of *in vitro* transcribed RNA was reversed upon the addition of the δ ag-S (Yamaguchi *et al.*, 2001). Together the above results suggest that the transcription and replication of HDV requires the δ ag-S to displace DSIF/NELF from RNAP II to allow for HDV RNA transcription and replication. The above study also suggests that RNAP II is required for HDV RNA transcription/replication. However, the above study does not provide direct evidence that HDV RNA transcription/replication is dependent on RNAP II.

1.4. Evidence that HDV RNA transcription/accumulation is dependent on DNA-dependent RNA polymerase II (RNAP II)

Direct evidence for the involvement of RNAP II for HDV RNA transcription/accumulation (HDV RNA accumulation refers to total HDV RNA that has accumulated over the course of replication, including RNA used to transfect, the mRNA generated from the genomic strand,

genomic and antigenomic HDV RNA transcripts) was initially reported when HDV RNA accumulation was shown to be sensitive to α -amanitin [a fungal toxin which in low concentrations is an inhibitor of RNAPII transcription, but not DNA-dependent RNA polymerase I (RNAP I) or DNA-dependent RNA polymerase III (RNAP III) transcription] (Macnaughton *et al.*, 1991; Fu and Taylor, 1993). However, HDV RNA accumulation was restored in the presence of cells containing an α -amanitin resistant allele of RNAP II, suggesting that HDV RNA accumulation is RNAPII-dependent (Macnaughton *et al.*, 1991; Fu and Taylor, 1993). A similar study later performed by Modahl and colleagues linked RNAP II to genomic, but not to antigenomic HDV RNA accumulation (Modahl *et al.*, 2000). They reported that genomic mRNA accumulation was inhibited by $<3\mu\text{g/ml}$ of α -amanitin, while the accumulation of full length (1.7kb) antigenomic HDV RNA was not affected by α -amanitin concentrations as high as $25\mu\text{g/ml}$ (Modahl *et al.*, 2000). Further studies revealed that following HDV RNA accumulation an equal amount of genomic HDV RNA was found in the nucleus and the cytoplasm, while antigenomic HDV RNA was retained in the nucleus (Macnaughton *et al.*, 2002). The proportions of genomic HDV RNA in the nucleus and the cytoplasm remained constant throughout replication, indicating that genomic HDV RNA is continuously exported out of the nucleus into the cytoplasm (Macnaughton *et al.*, 2002). The fact that antigenomic HDV RNA is not exported out of the nucleus, while genomic HDV RNA is continuously exported out of the nucleus, suggests that a selective export mechanism is required for genomic and antigenomic HDV RNA accumulation. Another investigation revealed that the $\delta\text{ag-L}$ inhibits genomic, but not antigenomic HDV RNA accumulation (Modahl and Lai, 2000). Together, these results suggest that a distinct

replication mechanism is required for genomic and antigenomic HDV RNA accumulation, indicating that genomic HDV RNA transcription is RNAPII-dependent while antigenomic HDV RNA transcription is RNAPII-independent (Modahl and Lai, 2000; Macnaughton *et al.*, 2002). The requirement of RNAPII for HDV RNA transcription and accumulation was further supported when the genomic mRNA was found to be post transcriptionally modified *in vivo* to contain a 5' cap and a 3' poly (A) tail (Hsieh *et al.*, 1990; Bichko *et al.*, 1996; Nie *et al.*, 2004; Gudima *et al.*, 2000). These post transcriptional modifications are unique to RNAPII transcripts (Egloff and Murphy, 2008), indicating that the transcription of the hepatitis *delta* antigen mRNA is RNAPII-dependent. The SC35, an essential nuclear ribonucleoprotein splicing factor, contains nucleolar subdomains that localize the protein to nuclear speckles (Bregman *et al.*, 1995). Nuclear speckles are active sites for RNAPII transcription (Bregman *et al.*, 1995). The SC35, an HDV interacting protein, co-localizes with the δ ag-S which interacts with HDV during HDV RNA accumulation (Hsieh *et al.*, 1990; Bichko *et al.*, 1996; Gudima *et al.*, 2000; Abraham and Pelchat, 2008; reviewed in Greco-Stewart and Pelchat, 2010). Together, these results all provide further support for RNAPII-dependent HDV RNA transcription and replication.

In vitro HDV RNA promoter studies were also conducted by various laboratories to provide direct evidence for the requirement of RNAPII for HDV RNA transcription. These studies also identified transcription initiation sites for HDV RNA transcription, and suggest that both polarities of HDV RNA possess RNA promoter activity *in vitro* (Beard *et al.*, 1996; Gudima *et al.*, 1999; Filipovska and Konarska, 2000; Lehmann *et al.*, 2007; Abraham and Pelchat, 2008). A study done by Beard and colleagues conducted an *in vitro* transcription assay with

HeLa nuclear extracts, and added 208nt derived from the right terminal stem loop domain of genomic HDV RNA (referred to as R199G throughout this thesis-Figure 1.1). This *in vitro* assay resulted in the transcription of a 100nt RNA transcript. Primer extension assays localized the initiation site to be at A₁₆₃₁ (Beard *et al.*, 1996). The A₁₆₃₁ is present upstream from the putative initiation site for the synthesis of the δ ag-S mRNA present at nucleotide U₁₆₃₀ (Gudima *et al.*, 1999). Experiments later refined the transcription start site to be at U₁₆₃₀ (Gudima *et al.*, 1999). This makes the first transcribed nucleotide an adenosine (A₁₆₃₀) which is a common feature of RNAP II transcripts (Gudima *et al.*, 1999). Thus, R199G was then referred to as a region with RNA promoter activity (Beard *et al.*, 1996; Gudima *et al.*, 1999). Recently, our laboratory performed an *in vitro* transcription assay utilizing HeLa nuclear extract as a source for RNAPII, and R199G as a template for transcription (Abraham and Pelchat, 2008). Following *in vitro* transcription, a reverse transcription (RT) was performed and the cDNA product was subjected to rapid amplification of the cDNA ends (RACE), which resulted in a 100-150nt PCR product. The RACE product was then sequenced to detect the transcription initiation site. The transcription initiation site was localized at U₁₆₃₀ which is in accordance with the previously reported transcription initiation site for the HDV *delta* antigen mRNA (Beard *et al.*, 1996; Gudima *et al.*, 1999). The location of the initiation site was further confirmed by utilizing a mutant form of R199G where the transcriptional initiation site was deleted. This did not yield an HDV RNA transcript, confirming the previously reported transcription initiation site at U₁₆₃₀ (Abraham and Pelchat, 2008). Next, an antibody against the CTD of RNAPII was used, which inhibited *in vitro* R199G transcription, while an electrophoretic mobility shift assay (EMSA)

demonstrated that purified RNAPII was directly binding to R199G. Thus, both assays indicated that R199G transcription is RNAPII dependent. Additional *in vitro* transcription assays revealed that a functional pre-initiation complex (PIC) can form on R199G, indicating that transcription initiation can be obtained with R199G (Abraham and Pelchat, 2008). Together, the above investigations all suggest that the mRNA transcribed from genomic HDV RNA is RNAP II dependent.

Other studies reported another HDV RNA promoter region located within the left terminal stem loop domain of the antigenomic strand (Filipovska and Konarska, 2000; Greco-Stewart *et al.*, 2007; Lehmann *et al.*, 2007). Filipovska and colleagues also utilized an *in vitro* transcription assay, but with the addition of a 280nt species derived from the left terminal stem loop domain of the antigenomic strand (Filipovska and Konarska, 2000). Their assay indicated that transcription of this species is sensitive to α -amanitin. The synthesis of this species can be partially restored using a nuclear extract from cells that contain an α -amanitin resistant allele of RNAPII. However, the RNA product was a chimeric species. This chimeric species was composed of a newly synthesized transcript covalently attached to the 5' end, which is derived from the left terminal stem loop domain of the antigenomic strand (Filipovska and Konarska, 2000). These results suggest that transcription in this system does not proceed via *de novo* RNAPII transcription. Rather, transcription occurred via cleavage of the RNA template followed by extension of the 3' end (Filipovska and Konarska, 2000). A more recent study done by Lehman and colleagues demonstrated that DNA-dependent RNAPII also possesses RNA-dependent RNA polymerase activity (Lehmann *et al.*, 2007). Lehman and colleagues revealed the crystal structure of purified RNAPII transcribing from

an RNA template derived from the tip of the left terminal stem-loop domain of the antigenomic strand of HDV RNA. They demonstrated that RNA-dependent RNAPII transcription is template driven and efficient but slower than that of DNA-dependent RNAPII transcription. The general transcription factor TFIIS, was speculated to cleave the terminal stem-loop region of HDV, to allow for the extension of the newly formed 3' end (Lehmann *et al.*, 2007). *In vitro* binding experiments done by our lab also confirmed that RNAPII specifically interacts with HDV RNA, within the terminal stem loop domains of both polarities of HDV RNA (Greco-Stewart *et al.*, 2007). Although, *in vitro* studies demonstrated that transcription of the left terminal stem loop domain of the antigenomic can serve as a template for RNAPII transcription, its role in HDV RNA replication remains questionable since transcription from this region occurs through the non-conventional 3'end extension mechanism (Filipovska and Konarska, 2000). Therefore, additional studies are required to determine the enzymatic requirements for transcription and accumulation from the antigenomic strand.

1.5. Evidence that HDV RNA transcription/accumulation is dependent on additional DNA-dependent RNA polymerases

More recent studies performed by the Lai laboratory provided direct evidence for the involvement of RNAP I for antigenomic HDV RNA transcription and accumulation (Li *et al.*, 2006; Chen *et al.*, 2008). The Lai laboratory reported that genomic HDV RNA accumulated throughout the nucleoplasm and is sensitive to α -amanitin (Li *et al.*, 2006), while antigenomic HDV RNA was accumulating in the nucleolus and was resistant to α -amanitin (Li *et al.*, 2006), consistent with their previous experiments (Modahl *et al.*, 2000). Further

experiments revealed that δ ag-S co-immunoprecipitated with RNAPII and a component of the transcriptional machinery of RNAPI, SL1 (Li *et al.*, 2006). Also, when *in vitro* transcription was performed on purified HDV particles in HeLa nuclear extracts, antigenomic HDV RNA transcription was inhibited when an α -SL1 antibody was used, whereas little effect was seen when an RNAP II antibody was used (Li *et al.*, 2006). Together, the above experiments provide evidence for the involvement of RNAPI for antigenomic HDV RNA accumulation.

More recent studies provided further evidence for the involvement of a distinct polymerase other than RNAPII for antigenomic HDV RNA accumulation. An experiment performed by Huang and colleagues utilized recombinant δ ag-S bound to a nucleolar localization signal, in order to solely localize it to the nucleolus (Huang *et al.*, 2008). This study reported that genomic HDV RNA accumulation was undetectable when recombinant δ ag-S was localized to the nucleolus, while antigenomic HDV RNA accumulation was unaffected, confirming that antigenomic HDV RNA accumulates in a distinct region within the nucleus (Huang *et al.*, 2008). They also found that genomic HDV RNA accumulation was restored when treated with actinomycin D, which allowed the nucleolar proteins to be distributed back to the nucleoplasm (Huang *et al.*, 2008) [actinomycin D is a general transcription factor that inhibits DNA-dependent RNA synthesis, but does not inhibit HDV RNA accumulation (Macnaughton *et al.*, 2002)]. Furthermore, RNAPII only co-immunoprecipitated with the δ ag-S when it was released to the nucleoplasm, however, RNAP I did not co-immunoprecipitate with the δ ag-S (Huang *et al.*, 2008) which is inconsistent with the previous studies performed by Li and colleagues (Li *et al.*, 2006). Additional studies are required to confirm whether antigenomic HDV RNA accumulation is dependent on the δ ag-

S. Recently our lab identified RNAPI and RNAPIII binding to both polarities of the terminal stem loop domains of HDV RNA *in vitro* (Greco-Stewart *et al.*, 2009).

Although, a few studies showed that antigenomic HDV RNA accumulation is RNAP I dependent, a more recent study performed by the Taylor laboratory (Chang *et al.*, 2008) suggested otherwise. They reported that both polarities of the HDV RNA transcripts were reduced to ~14%, when pre-treated with low concentrations of α -amanitin, while transcripts of RNAPI and III were unaffected. The Taylor laboratory, and our laboratory demonstrated that both polarities of unit length HDV RNA interacted with complexes of RNAPII (Greco-Stewart *et al.*, 2007; Chang *et al.*, 2008) and the δ ag-S in HDV replicating cells (Chang *et al.*, 2008). Furthermore, both RNAPII and the δ ag-S co-localized to the nucleoplasm, which replicates HDV RNA, indicating that transcription was occurring (Chang *et al.*, 2008). Therefore, these findings suggest that HDV RNA accumulation is most likely RNAPII dependent.

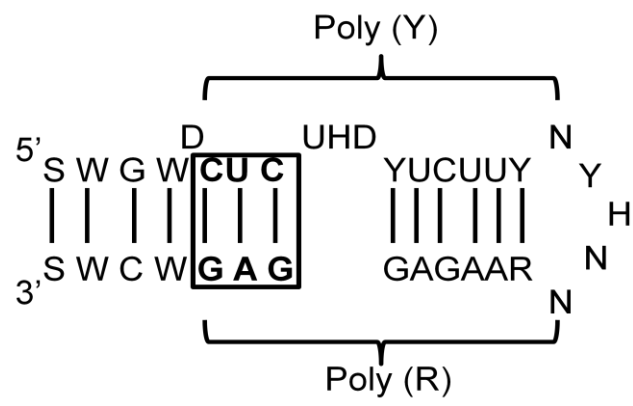
Although, HDV RNA accumulation might also require RNAPI and III, and both polymerases might select their RNA templates in a similar manner to RNAPII, the focus of this study is understanding how RNAPII selects an RNA promoter for transcription.

1.6. Structural characteristics of HDV RNA promoters

Several structural/nucleotide features are required in order for HDV RNA to be recognized by RNAP II for HDV RNA replication/accumulation to occur. A bioinformatics study conducted on 81 isolates of HDV was performed by our lab, on both polarities of the terminal stem loop domains of HDV RNA shown to specifically interact with RNAPII *in vitro* (Greco-Stewart *et al.*, 2007). This study investigated the structural features required by

RNAPII to interact with HDV RNA (Greco-Stewart *et al.*, 2007). Analysis of base-pair co-variation indicated that changes in the primary sequence of the terminal stem loop domains might be tolerated for RNAPII binding to HDV RNA, but the secondary structures are critical for recognition by RNAPII to allow for HDV RNA replication/accumulation. The secondary structure located at the tip of the rod, is comprised of double stranded RNA regions with a conserved CUC/GAG triple base-pair motif, one strand of pyrimidine located upstream a 3-8nt terminal loop which is followed by a complementary strand of purines. (Figure 1.4 represents this secondary structure obtained from the right terminal stem loop domain of the genomic strand). The conformation and the polarization of the purine/pyrimidine structures located at both ends of the rod are required for recognition by RNAP II. This is because when mutations were introduced at either end of the rod like structure, aimed at mutating either of the conserved purine/pyrimidine to pyrimidine/purine structures, no significant association of RNAPII was found with either of the mutants *in vitro* (Greco-Stewart *et al.*, 2007). Several other studies have designed various HDV RNA mutants that were transfected in HeLa cells, and RNA was extracted and quantified via northern analysis to measure the difference in HDV RNA accumulation following transfection with the mutants (Chao *et al.*, 1990; Beard *et al.*, 1996; Wang *et al.*, 1997; Wu *et al.*, 1997; Gudima *et al.*, 1999; Filipovska and Konarska, 2000; Gudima *et al.*, 2006). One such study found that when the overall rod like structure was disrupted, this resulted in no HDV RNA accumulation (Beard *et al.*, 1996). Other studies found that the region containing the *delta* ribozyme motifs, and encoding the *delta* antigens in the HDV rod like structure, is always

Figure 1.4: Schematic representation of the secondary structure of the right terminal stem loop domain of the genomic strand in HDV RNA. Secondary structure were derived by analysis of 81 HDV isolates. The conserved CUC/GAG, Polypyrimidine (Poly Y) and Polypurine (Poly R) motifs are indicated. The IUPAC-letter code abbreviations were used to identify the nucleotides on the secondary structure (Figure adapted from Greco-Stewart *et al.*, 2007; Figure 4B).



biologically selected for during HDV RNA replication (Chao *et al.*, 1990; Gudima *et al.*, 2006). For instance, the insertion of a large number of nucleotides in this region disrupts the secondary rod like structure which is not tolerated. This results in the reversion to the rod like structure with ~1700nt (Chao *et al.*, 1990; Gudima *et al.*, 2006). HDV RNA accumulation is also dependent on the right terminal stem loop domain of the genomic strand (R199G). When HDV RNA mutants were constructed to contain large deletions at the R199G region, HDV RNA accumulation did not occur (Lazinski and Taylor, 1994).

Another region required for HDV RNA accumulation resembles an external bulge which contains 3nt (Figure 1.5 A) located within the extreme tip of R199G. This site contains the putative transcriptional start site (U₁₆₃₀/A₁₆₃₁). Mutations at this site result in significantly lower HDV RNA accumulation when compared to the control (Beard *et al.*, 1996; Wang *et al.*, 1997; Gudima *et al.*, 1999). As for the antigenomic strand, the transcriptional start site was localized within the extreme tip of the hairpin structure. Disruption of the 2nt (Figure 1.5 B) of the external bulge, located next to the putative transcription initiation site on the left terminal stem loop domain of the antigenomic strand, results in significantly lower HDV RNA accumulation when compared to the control (Filipovska and Konarska, 2000).

Another important aspect for RNAPII HDV RNA promoter recognition are the 5nt at the terminal loops of both polarities attached to a 6nt stem (Figure 1.5) (Beard *et al.*, 1996; Wu *et al.*, 1997; Gudima *et al.*, 1999). Mutations at the 5nt site located at the terminal stem loops which substantially alter the secondary structure, result in no HDV RNA accumulation. HDV RNA accumulation is slightly affected when the nucleotides within the loops are randomized or two to four nucleotides are added to the terminal loops (Beard *et al.*, 1996;

Figure 1.5: Extreme terminal domains of HDV RNA. Extreme tip of the terminal domains of the a) right genomic polarity, reported to possess promoter activity *in vitro* (Beard et al., 1996; Abraham and Pelchat, 2008) and b) left antigenomic polarity also reported to possess promoter activity *in vitro* (Filipovska and Konarska, 2000; Lehman *et al.*, 2007). External bulges are outlined to depict putative transcriptional start sites for genomic and antigenomic polarities (Figure adapted from Greco-Stewart *et al.*, 2007; Figure 4B).

Wu *et al.*, 1997; Gudima *et al.*, 1999). Also when the 6nt present within the stem of both terminal loops are deleted, HDV RNA accumulation does not occur. HDV RNA accumulation was significantly lower in comparison to the control when 1-3nt were added to the 6nt regions (Beard *et al.*, 1996; Wu *et al.*, 1997; Gudima *et al.*, 1999). This indicates that both the stem loop structures are important for stabilizing the ~1,700nt secondary rod like structure (Beard *et al.*, 1996; Wu *et al.*, 1997; Gudima *et al.*, 1999). Together, these results suggest the maintenance of the secondary structure is more important than the nucleotide compositions for RNAPII HDV RNA promoter recognition.

However, it is important to note that the HDV terminal stem loop domains might also be associated with other cellular processes unrelated to RNAPII binding. Such processes include RNA stabilization, export/import, and RNA encapsidation (Greco-Stewart *et al.*, 2007).

1.7. Host proteins that interact with HDV RNA

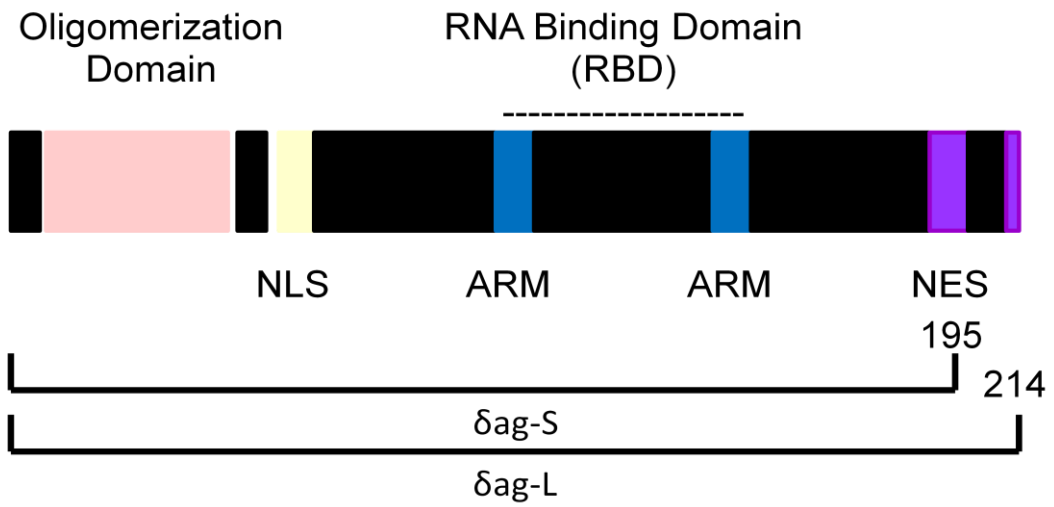
Characterizing the host proteins that interact with HDV RNA will help characterize how RNAPII selects its RNA promoter. 'Interactions' also refer to indirect binding between proteins that might occur through other proteins.

An HDV interacting protein is the adenosine acting on RNA (ADAR-1) (Wong and Lazinski, 2002). ADAR-1 changes the adenosine to inosine in the UAG amber termination codon for the δ ag-S resulting in a new codon formation UIG, where inosine is read as guanine resulting in a codon read as tryptophan (Wong and Lazinski, 2002). Because this modification occurs on an RNA that can be processed into the antigenomic strand, it is therefore kept in the remaining rounds of replication until it finally gets incorporated in the

δ ag-S mRNA resulting in a translational read-through. This translation generates a 19-amino acid larger sequence relative to the δ ag-S. These 19-amino acids are located at the C-terminus, resulting in the production of the δ ag-L (Wong and Lazinski, 2002). However, the large form of the HDV *delta* antigen does not have supporting functions for genome replication. The δ ag-L has been shown to be a dominant negative inhibitor of replication (Chao *et al.*, 1990; Macnaughton and Lai, 2002). The δ ag-L on its own can bind to the hepatitis B surface antigens for viral packaging, but the presence of the δ ag-S enhances this process (Wang *et al.*, 1994). The structural characteristics of both antigens distinguish their roles apart during HDV replication. Both antigens contain a nuclear localization signal (NLS- Figure 1.6), the leucine rich coiled-coil domain required for the oligomerization of the antigens, and two arginine rich motifs required for RNA binding, and import/export (reviewed in Greco-Stewart and Pelchat, 2010). The N-terminal domain of the δ ag-S possesses RNA chaperone activity by promoting the annealing of various complimentary sequences and stabilizing RNA duplexes (Huang *et al.*, 2003). Others have demonstrated that both the δ ag-S and δ ag-L serve to stabilize HDV RNA (Lazinski and Taylor, 1994) and enhance ribozyme activity (Jeng *et al.*, 1996). The 19 amino acid residues on the C-terminal domain of the δ ag-L, along with the coiled-coil dimerization domain (within the oligomerization domain) are both required to bind to the δ ag-S to inhibit HDV replication (reviewed in Greco-Stewart and Pelchat, 2010).

On the other hand, the function of the C-terminal Pro/Gly-rich region in both antigens remains unclear (Macnaughton and Lai, 2002). The end of the C-terminal domain in the

Figure 1.6: Schematic representation of the HDV antigens. The δ ag-S spans amino acids 1-195, while the δ ag-L spans amino acids 1-214. The HDV antigens contain the following functional domains: the nuclear localization signal (NLS), the nuclear export signal (NES), the arginine rich motifs (ARMs) and the RNA binding domain (RBD) which spans the two arginine rich motifs and the coiled-coil oligomerization domain. (Figure adapted from Greco-Stewart and Pelchat, 2010; Figure 2)



δ ag-L has four amino acid motifs (CXXQ) where prenylation occurs at the cysteine residue (Glenn *et al.*, 1992; Lee *et al.*, 1994; Otto and Casey, 1996). Prenylation causes a conformational change that masks the carboxyl terminal domain (CTD) of δ ag-L (Hwang and Lai, 1994). This allows the δ ag-L to interact with the HBV surface antigens a critical step in HDV particle assembly (Lee *et al.*, 1994). The δ ag-L contains a nuclear export signal (NES) required to export HDV RNA out of the nucleus following replication (reviewed in Greco-Stewart and Pelchat, 2010). The RNA binding domain (RBD) is another domain of interest (Lin *et al.*, 1990; Chao *et al.*, 1991). The RBD is encompassed of two arginine-rich motifs (ARM) that are separated by a spacer region. This spacer region contains the helix-loop-helix (HLH) motif (Lee *et al.*, 1993). Both the NLS and the ARM are required for the entry of HDV RNA to the nucleus following an HDV infection (Chang *et al.*, 1995). The ARM motifs in addition to the HLH motifs are both essential for RNA binding and enhancing HDV RNA accumulation *ex-vivo* (Lee *et al.*, 1993). However, these motifs can be deleted from the δ ag-L without compromising its functional abilities (Lazinski and Taylor, 1993).

Protein kinase R (PKR) is another host HDV interacting protein. Studies have demonstrated the ability of host double stranded RNA-specific proteins such as PKR, to interact with single stranded RNA molecules and be activated by them (Robertson *et al.*, 1996). Thus, when PKR is activated interferon antiviral responses are induced. These interferons act as tumor suppressors by inhibiting cellular translation (Robertson *et al.*, 1996). The unbranched rod like structure of HDV is the substrate for activation of PKR (Robertson *et al.*, 1996; Circle *et al.*, 1997). When HDV interacts with PKR, PKR phosphorylates the *delta* antigens. Although, PKR is activated when it binds HDV, it is unable to inhibit protein synthesis (Robertson *et al.*,

1996). It is proposed that PKR is unable to inhibit protein synthesis, possibly due to the interaction of HDV with PKR, to inhibit its ability to induce interferon response. Or it could be that HDV RNA interacts with PKR to recruit it to the RNP complex containing the HDV *delta* antigens, which can then be phosphorylated by PKR. However, this theory is yet to be tested.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is another cellular protein that has been found to interact with HDV RNA (Sikora *et al.*, 2009) to enhance *delta* ribozyme activity (Lin *et al.*, 2000). It is thus proposed that GAPDH enhances HDV RNA replication by acting as a molecular chaperone for the unwinding of HDV RNA to allow for autocatalytic cleavage during replication (Sioud and Jespersen, 1996; Lin *et al.*, 2000).

A study done by our lab (Sikora *et al.*, 2009) identified various other proteins found to interact with the putative promoter region of the right terminal stem loop domain of HDV RNA (R199G). Proteins include the eukaryotic elongation factor 1A1 (eEF1A1), the 54kDa nuclear binding protein (p54^{nrb}), the heterogeneous nuclear ribonucleoprotein L (hnRNP-L), and the alternative splicing factor (ASF/SF2) (Sikora *et al.*, 2009). To date the functions of eEF1A1, p54^{nrb} and ASF/SF2 remain unknown in the HDV life cycle.

The protein of interest in my thesis is the polypyrimidine tract-binding protein associated splicing factor (PSF). *In vitro* UV-crosslinking assays identified PSF as an HDV interacting protein (Greco-Stewart *et al.*, 2006). Identified by Patton and colleagues, PSF is a 76kDa multifunctional protein (Figure 1.7) that forms a part of the polypyrimidine tract-binding protein (PTB) complex (Patton *et al.*, 1993). This multifunctional protein, also known as the

Figure 1.7: Schematic representation of the polypyrimidine tract-binding protein associated splicing factor (PSF). PSF is a 76kDa multifunctional protein that forms a part of the polypyrimidine tract-binding protein (PTB) complex. The domains that make up PSF include the C-terminus RNA recognition motifs (RRM1 and RRM2) and the highly charged domain (+/-). The N-terminus contains the proline (P), glutamine (Q) rich domains and, the arginine (R), glycine (G) rich domains (RGG) (Figure adapted from Shav-Tal and Zipori, 2002; Figure 1).



splicing factor proline/ glutamine-rich (SFPQ), can act separately or form a heterotetramer with the 54kDa p54^{nrb} (Dong *et al.*, 1993). Both PSF and p54^{nrb} belong to a family of proteins that share close homology within a 246 amino acid central conserved region. This conserved region contains two RNA recognition motifs (RRM) and a homo/heterodimerization domain (Dong *et al.*, 1993). The p54^{nrb} protein has high homology to the CTD domain of PSF. Both these proteins have been shown to bind single and double stranded DNA and RNA molecules (Shav-Tal and Zipori, 2002). PSF has been shown to be involved in many cellular functions including pre-mRNA splicing, polyadenylation, transcriptional regulation, nucleic acid unwinding and pairing, retention of defective RNAs, and nuclear transport (Shav-Tal and Zipori, 2002). PSF has also been localized in the nuclear matrix, the nucleoplasm and paraspeckles (Shav-Tal and Zipori, 2002).

As described above, the eukaryotic RNAPII responsible for the synthesis of messenger RNA (mRNA) (reviewed by Cramer, 2002; Cramer *et al.*, 2008) is another HDV interacting protein. RNAPII contains 10 core subunits: Rpb1, Rpb2, Rpb3, Rpb5, Rpb6, Rpb8, Rpb9, Rpb10, Rpb11 and Rpb12 (Cramer *et al.*, 2001). The CTD is part of the largest RNAPII subunit Rpb1. The human CTD is composed of 52 heptapeptide repeats (YSPTSPS) (reviewed in Egloff and Murphy, 2008). These consensus repeats are subject to site specific phosphorylation of tyrosine and serine, and glycosylation of threonine and serine (reviewed in Egloff and Murphy, 2008). The phosphorylation of the serine on the CTD is essential for the selection of certain transcription factors that can interact with RNAPII during transcription of an RNA product derived from a DNA template. Phosphorylation of the serine residues at positions 2 and 5 have been associated with transcriptional elongation, and 5' capping of the mRNA,

respectively (Meinhart *et al.*, 2005; reviewed in Egloff and Murphy, 2008). Dephosphorylation of these serine residues results in the recycling of RNAPII (Komarnitsky *et al.*, 2000). The hyper and hypophosphorylated conformations of the CTD of RNAPII are associated with transcription elongation and initiation, respectively (reviewed Egloff and Murphy, 2008).

Studies have reported that PSF might interact with the CTD of RNAPII during transcription. This was demonstrated in previous *in vitro* co-immunoprecipitation assays which reported that PSF interacts with hypo and hyperphosphorylated forms of the CTD domain of RNAP II (Emili *et al.*, 2002). This experiment was performed by co-immunoprecipitation assays using HeLa nuclear extracts, as a source of PSF and RNAPII, and the monoclonal antibodies 8WG16 and B3. The 8WG16 and B3 antibodies were used in the co-immunoprecipitation experiments, since they have been previously shown to specifically interact with hypo or hyperphosphorylated forms of the CTD of RNAP II, respectively (Thompson *et al.*, 1990; Mortillaro, *et al.*, 1996). PSF was also reported to facilitate the interaction of RNA transcripts with the CTD of RNAPII (Emili *et al.*, 2002). This experiment utilized immobilized glutathione *S* transferase (GST)-CTD columns which were pre-incubated with or without purified PSF, and allowed to bind to radiolabeled RNA transcripts. The enhanced ability of the GST-CTD column containing highly purified PSF to retain the RNA transcripts compared to the column not containing PSF suggested that PSF might have a role in associating RNA with the CTD of RNAPII (Emili *et al.*, 2002). Together, these results suggest that PSF associates the RNA with the CTD of RNAPII. PSF allows for this association by interacting with the CTD of RNAPII during transcription initiation and elongation (Emili *et al.*, 2002).

In order to verify whether PSF can interact with HDV RNA, our lab performed a series of co-immunoprecipitation assays utilizing HeLa nuclear extract which contained transfected genomic and antigenomic HDV RNA (Greco-Stewart *et al.*, 2006). The co-immunoprecipitation assay was performed with an antibody raised against PSF, which determined that PSF co-immunoprecipitated with both polarities of HDV RNA. The same experiment was repeated, but utilizing the following radiolabeled HDV derived RNAs expressing only one of the following sequences: the right terminal stem loop domain of the genomic or antigenomic polarity, the left terminal stem loop domain of the genomic or antigenomic polarity, or the central region of the genomic or antigenomic polarity. These HDV RNAs were also subjected to an EMSA with purified recombinant PSF (Greco-Stewart *et al.*, 2006). Both experiments revealed that PSF interacted with both polarities of the terminal stem loop domains of HDV RNA (Greco-Stewart *et al.*, 2006) also shown to interact with RNAPII *in vitro* (Greco-Stewart *et al.*, 2007). Thus, based on the experiments performed by (Emili *et al.*, 2002) and our lab, it was then proposed that PSF might act as a transcription factor by recruiting RNAPII to HDV RNA during HDV RNA transcription initiation (Greco-Stewart thesis, 2009).

In order to determine whether PSF is required for HDV to interact with RNAPII, a series of *in vitro* competition co-immunoprecipitation assays were performed by our lab. The competition experiments verified whether PSF-HDV RNA and PSF-RNAPII interactions are essential for HDV RNA-RNAPII interactions. HeLa nuclear extracts were used in the presence of an α -RNAPII (8WG16) antibody (Greco-Stewart thesis, 2009). Since there are no specific PSF inhibitors, the SELEX (systematic evolution of ligands by exponential enrichment)

derived-RNA approach was used. The SELEX RNAs that were chosen were two PSF binding sequences (S1 and S17) and a PSF binding trimeric consensus repeat (Peng *et al.*, 2002). When an excess of these competitor RNAs were added to the co-immunoprecipitation reaction, R199G was unable to co-immunoprecipitate with the RNAPII complex. This indicates that PSF has to interact with HDV RNA (R199G) in order for HDV to interact with RNAPII *in vitro* (Greco-Stewart thesis, 2009). Further experiments were performed to test whether the PSF-RNAPII interaction is required for HDV-RNAPII interaction. An excess of the purified CTD peptide of RNAPII was added to the co-immunoprecipitation reaction, which competes with the RNAPII-PSF interaction. The addition of excess CTD prevented R199G from co-immunoprecipitating with RNAPII. This suggests that PSF has to interact with the CTD of RNAPII in order for HDV to interact with RNAPII (Greco-Stewart thesis, 2009).

Because the above experiments demonstrated that PSF is required for the binding of HDV RNA to RNAPII, further experiments were done in order to test whether both PSF and RNAPII are simultaneously interacting with HDV RNA (Greco-Stewart thesis, 2009). This experiment was essential for verifying whether PSF might be acting as a transcription factor during RNAPII HDV RNA transcription. Thus, RNA affinity chromatography was used since it determines what makes up the complex that forms on HDV RNA (R199G). Following the formation of the R199G complex on streptavidin beads, HeLa nuclear extract a source of both PSF and RNAPII was added to the reaction and the eluted complex was subjected to a co-immunoprecipitation assay. The co-immunoprecipitation assay used either α -PSF or α -RNAPII to co-immunoprecipitate PSF or RNAPII, respectively. The eluted protein complexes were then subjected to western blot analyses, in order to detect whether RNAPII also co-

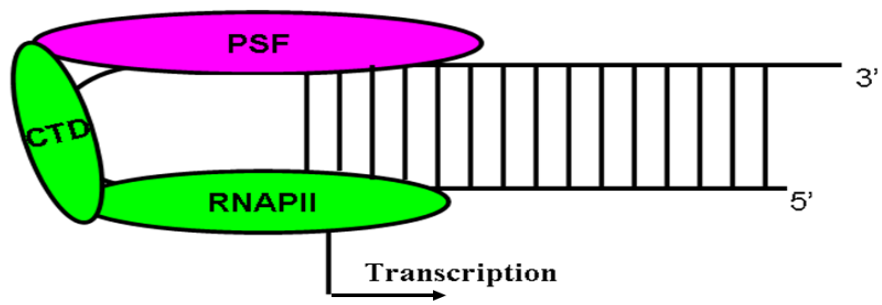
immunoprecipitated with PSF or vice versa. Because RNAPII co-immunoprecipitated with PSF and vice versa, it was concluded that both PSF and RNAPII interacted with R199G simultaneously and this interaction was proven to be specific to R199G (Greco-Stewart thesis, 2009).

1.8. Statement of purpose and hypothesis

Previous experiments demonstrated that PSF interacts with both hypo and hyper-phosphorylated conformations of the CTD of RNAPII, and facilitates the interaction of RNA transcripts with the CTD of RNAPII (Emili *et al.*, 2002). PSF and RNAPII were then found to interact with the right and left terminal stem loop domains of the genomic and antigenomic HDV RNA; two putative RNA promoter regions (Greco-Stewart *et al.*, 2006; Greco-Stewart *et al.*, 2007). PSF-HDV interaction was required for HDV to interact with RNAPII, and both PSF and RNAPII were found to simultaneously interact with HDV RNA (Greco-Stewart Thesis, 2009). Thus, all the above experiments suggest that PSF might be required for RNAPII dependent HDV RNA transcription. In order to further investigate the relationship of PSF to HDV RNA accumulation, the objectives of my thesis were to determine whether PSF affects *ex-vivo* HDV RNA accumulation, determine which PSF domains are required for HDV RNA accumulation, and determine whether these domains are required to interact with RNAP II *in vitro*. Therefore, my thesis was aimed at providing further insight in how DNA-dependent RNAPII selects an RNA promoter to transcribe from an RNA template based on the following hypothesis:

PSF acts as a transcription factor during HDV RNA replication by interacting with both the CTD of RNAPII and HDV RNA simultaneously (Figure 1.8).

Figure 1.8: Schematic representation of my proposed hypothesis. PSF acts as a transcription factor during HDV RNA replication by interacting with both the Carboxy terminal domain (CTD) of RNAPII and HDV RNA simultaneously.



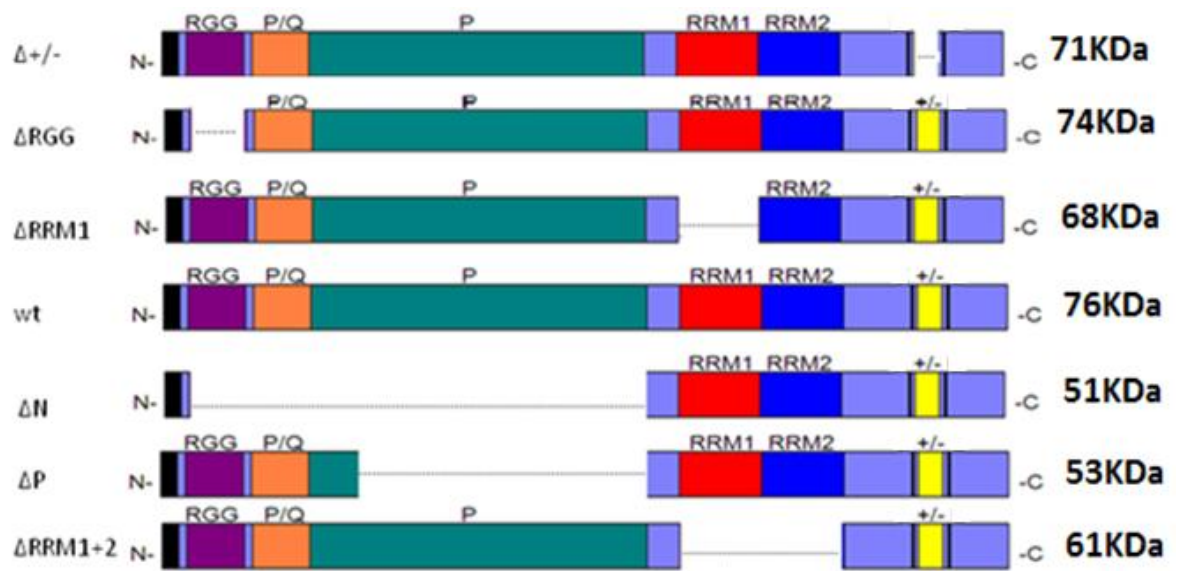
In order to link PSF to HDV RNA accumulation, the objectives of the present study were thus:

- To determine the physiological significance of PSF in relation to HDV RNA. This experiment involved knocking down PSF in HDV replicating cells. This helped determine whether PSF had an effect on HDV RNA accumulation *ex-vivo*.

To identify the PSF domains that were required for HDV RNA accumulation. This experiment determined the PSF domains that were required to affect **basal levels and the overproduction** of HDV RNA accumulation. Thus, HDV replicating cells were transfected with N- terminal C-myc-epitope tagged deletions derivatives of PSF (Figure 1.9) that were constructed by Rosonina and colleagues by specifically removing the RGG box (Δ RGG residues 9 to 27), RRM1 (Δ RRM1 residues 298 to 365) and the basic/acidic rich domain +/- (Δ +/- residues 575 to 611). More extensive deletions were also constructed, which removed the entire N terminal half of PSF (Δ N residues 9 to 264), the proline rich region (Δ P residues 26 to 267) and both RRMS (Δ RRM1+2, residues 298 to 429) (Rosonina *et al.*, 2005).

- To identify the PSF domains that were required to interact with RNAPII *in vitro*, by determining which PSF mutants interacted with RNAPII.

Figure 1.9: Schematic representation of the myc epitope tagged wild type and mutant PSF proteins. Diagram showing the domain organizations in PSF, and the regions that have been removed in the different PSF mutant expression plasmids (Figure adapted from Rosonina *et al.*, 2005 Figure 4A).



2.0 Materials and Methods

2.1. Transfections

The myc epitope tagged wild type and mutant PSF plasmids were generously provided to us by Dr. Benjamin Blencowe (University of Toronto). In order to transfect the HEK293 cells with 3µg of myc-epitope wild type or mutant PSF plasmids, I needed to overproduce them in bacteria (*E.coli*) and purify them as previously described (Rosnina *et al.*, 2005) with slight modifications to the protocol. Briefly, *E.coli* K-12 cells containing the DNA of interest were streaked on 10cm petri dishes containing Lysogeny broth (LB) with 100µg/mL of ampicillin and incubated overnight at 37°C. Individual colonies were selected and inoculated overnight in 5mL of LB containing 100µg/mL of ampicillin at 37°C. The plasmids were purified using the Qiaprep kit Miniprep kit (Qiagen), and quantified using spectrophotometry at 260nm. For the transfection of the HEK293 cells, lipofectamine 2000 (Invitrogen) was used with slight modifications to the manufacturers protocol. Briefly, approximately 1×10^6 cells were seeded in 6 well plates coated with gelatine (Corning) using Dulbecco's modified eagle serum (DMEM-invitrogen) with 10% fetal bovine serum (FBS) and incubated overnight in a 5% CO₂ incubator at 37°C. Transfections were carried out once the cells were 70% confluent. Transfections were performed using 12µL of lipofectamine 2000 suspended in 200µL DMEM (Invitrogen) incubated at room temperature for 5min. 3µg of each plasmid were re-suspended in another 200µL of DMEM. Following incubation, the mixtures were combined and mixed by pipetting and left to incubate at room temperature for 30min. Once the mixtures were ready, they were added drop-wise to the corresponding wells. The plates were then rocked back and forth and incubated at 37°C in a 5% CO₂

chamber. The HEK293 cells were harvested 48 hours post transfection, and cell lysate was collected using the following method; media in each well was aspirated, and the cells were washed once with 1X phosphate buffered saline (PBS) (Invitrogen). The cells were trypsinized using 500 μ L of trypsin 0.25% EDTA (Invitrogen). The cells were collected in individual microcentrifuge tubes and spun at 1500rpm for 5 min at 4°C using the Sorvall microcentrifuge (Fisher). The supernatant was aspirated, and the cell pellet was washed twice with 1mL 1X PBS and spun down at 1500rpm at 4°C between each step. The cell pellet was re-suspended by pipeting in 60 μ L of cell lysis buffer (1% protease inhibitor cocktail 1, 1% phosphatase inhibitor cocktail 2, 1mM pmsf, 50mM Tris-HCl, pH 7.5, 1% Nonidet p-40 (NP-40), 0.5% sodium deoxycholate, 0.05% SDS, 1mM EDTA, 150mM NaCl). The cell lysate was sonicated twice for 2 second intervals and incubated on ice for 10 seconds between each interval. 20% glycerol was added to the cell lysate prior to storage at -80°C.

The other human cell line HEK293- δ ag-S used in my thesis was derived from HEK293 cells. The HEK293- δ ag-S cells were previously prepared by (Chang *et al.*, 2005a). Chang and colleagues stably transfected HEK293 cells with a single copy of cDNA encoding the δ ag-S. The expression of the δ ag-S is under the control of a tetracycline (TET) promoter. Mutated HDV RNA generated from deleting 2-nucleotides located at the *Eco*RI site (a change that interferes in the translation of the δ ag-S) was then transfected in order to achieve HDV RNA replication in HEK293- δ ag-S cells. Therefore, the integrated cDNA copy of the δ ag-S is the only unchangeable source for the δ ag-S protein. In order to maintain the δ ag-S and TET repressor genes, HEK293- δ ag-S cells were maintained in DMEM with high glucose, 10 % fetal bovine serum (FBS) and the selection antibiotics 200 μ g/mL hygromycin and 5 μ g/mL

blasticidin (Invitrogen). In the absence of TET, basal levels of HDV are produced to allow for HDV replication which can be maintained for approximately 1 year (Chang *et al.*, 2005b). The HEK293- δ ag-S cells were transfected with myc epitope tagged wild type and PSF mutants as described above. For the purposes of over producing the δ ag-S, I waited 24 hours post transfection to induce the δ ag-S using 1 μ g/mL of tetracycline, suspended in 10mL of DMEM containing 10% FBS with the appropriate selection antibiotics 200 μ g/mL hygromycin and 5 μ g/mL blasticidin (Invitrogen) (Chan *et al.*, 2005a). The cells were harvested 48 hours post transfection. Total RNA and protein were extracted using Trizol (Invitrogen).

2.2. Western blot experiments

Following protein extractions, equal volumes of all protein extracts obtained from HEK293- δ ag-S cells were subjected to a 10% Sodium dodecyl sulphate (SDS)-PAGE [separating gel: [Acrylamide:Bis Acrylamide (19:1)], 1.5M Tris HCl pH 8.8, 10% (SDS), 10% Ammonium Persulfate (APS), 0.5% TEMED], the stacking gel [Acrylamide:Bis Acrylamide (19:1)], 0.5M Tris HCl pH 6.8, 10% SDS, 10% APS, 0.5% TEMED] run in 1XSDS buffer (25mM Tris-HCl, 200mM Glycine, 0.1% SDS). Once the SDS-PAGE finished running, proteins were subjected to an overnight transfer at 4°C at 25V on a nitrocellulose membrane in 1X transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Following the protein transfer, the western membrane was blocked for 1 hour with agitation using 3% non fat milk in 1X Tris buffered saline (TBS). The blocking solution was then exchanged with a primary antibody solution (1:500) in 3% non fat milk and left to incubate overnight at 4°C with agitation. The following day, the membrane was washed 3 times for 10min in 1XTBS, and incubated for 2 hours at

room temperature with agitation in 3% non fat milk containing 1:10000 secondary rabbit polyclonal antibody (raised against mouse IgG-HRP conjugated-Abcam). The membrane was then washed 3 times with 1X TBS for 10min at room temperature with agitation, followed by a 4 minute exposure using the chemiluminescence substrate SuperSignal West Pico Substrate (Fisher). Where indicated, a separate SDS-PAGE /protein transfer was performed for the detection of the Myc tag (α -myc clone 9E10-raised against c-Myc of Myc-Abcam), or β -Actin (α - β -Actin clone Ac-15- raised against the N-terminal β isoform of Actin-Abcam) or PSF (α -SFPQ clone B92-raised against PSF-VWR).

2.3. Ribonucleoprotein immunoprecipitation assay (RIPA)

The RIPA assay was performed as previously described in (Niranjanakumari *et al.*, 2002) using the protein G immunoprecipitation kit (Sigma Aldrich) with slight modifications. Approximately, 5×10^6 cells were used to prepare cell lysate obtained from HEK293 cells, transfected with $3 \mu\text{g}$ of wild type or mutant PSF plasmids (described above). Total amounts of isolated protein were quantified using the Bradford Assay (Bio Rad). Where indicated, the cell lysate was pre-treated with 10 (u)/ $50 \mu\text{g}$ of RNase A (Qiagen) and 10 (u)/ $50 \mu\text{g}$ of DNase I (Invitrogen). Following the pre-treatment, $50 \mu\text{g}$ of total cell lysate were incubated with $6 \mu\text{g}$ of α -RNA polymerase II (clone 8WG16- raised against the CTD of RNAPII-Cedarlane) or $6 \mu\text{g}$ of α -IgG (Goat polyclonal anti-rabbit-Abcam), suspended in 1X RIPA buffer (50mM Tris-HCl, pH 7.5, 1% Nonidet p-40 (NP-40), 0.5% sodium deoxycholate, 0.05% SDS, 1mM EDTA, 150mM NaCl) in a $100 \mu\text{L}$ total reaction volume. The samples were incubated for 2 hours at 4°C with agitation. Following the 2 hour incubation at 4°C , $30 \mu\text{L}$ of pre-washed protein G sepharose beads (Sigma Aldrich) were added to each reaction and

incubated at 4°C overnight with agitation. Following the overnight incubation the beads were washed 5 times with 700µL of 1X RIPA buffer. Protein samples were heated at 95°C for 5 min using 40µL of 1X SDS loading buffer (63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue, pH 6.8). The eluates were collected by centrifugation for 1 min at 13,000 g. The eluted proteins were subjected on a 10% polyacrylamide SDS-PAGE, followed by a western blot to detect the Myc tag.

2.4. Knockdown experiments

For the purpose of knocking down PSF, 1×10^6 Hek293- δ ag-S cells were seeded in 6 well plates and transfected at 80% confluency using a mixture of 3 siRNAs [PSF siRNA (h) catalogue number: sc-38304-Santa Cruz]. The siRNAs consist of 20-25nt designed to specifically knock down PSF gene expression. Knockdown controls included mock treatments and 5 scrambled siRNA's [siRNA (a) catalogue number: sc-37007-Santa Cruz]. According to the manufacturer, the scrambled controls contain a mixture of siRNA scrambled sequences that are not known to target any cellular mRNA. The manufacturers protocol (Santa Cruz) was followed with minor modifications. Briefly, for each transfection two mixtures were prepared. Mixture A contained 6µL of siRNA duplex diluted in 100µL of siRNA transfection medium (Santa Cruz). Mixture B contained 6µL of siRNA transfection reagent (Santa Cruz) diluted in 100µL of transfection medium. For the mock treatment, mixture A contained 116µL of siRNA transfection medium, and Mixture B contained 6µL of siRNA transfection reagent diluted in 100µL of transfection medium. Both mixtures A and B were combined and mixed by pipetting. The mixture was incubated for 30 min at room temperature and 0.8mL of siRNA transfection medium was added to each tube containing

mixture A and B, and mixed by pipetting. The cells were washed with 2mL siRNA transfection medium, and the mixture was added dropwise to the washed cells, and incubated for 7 hours at 37°C in a 5% CO₂ incubator. The medium was then changed with DMEM medium containing 10% FBS with the appropriate selection antibiotics hygromycin and blasticidin (Invitrogen) to maintain the δ ag-S and TET repressor genes (Chan *et al.*, 2005). 24 hours post transfection the expression of the δ ag-S was induced with 1 μ g/mL of TET. 48 hours post transfection the cells were harvested. Total RNA and Protein were extracted using Trizol (Invitrogen). All experiments were done in triplicates. All protein extracts were subjected to a 10% polyacrylamide SDS-PAGE, followed by western blot analyses for PSF and β -Actin.

2.5. Reverse transcription (RT)-Quantitative Polymerase Chain Reaction (qPCR)

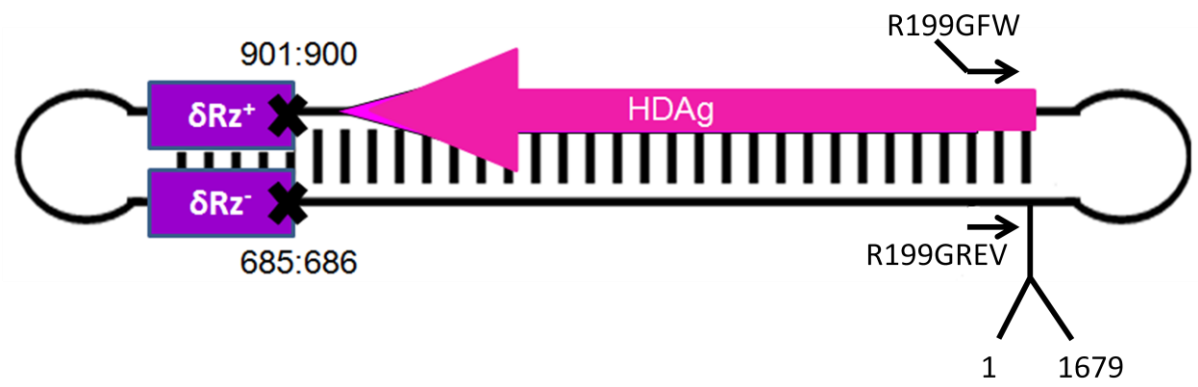
Following RNA purification using Trizol (Invitrogen), total RNA obtained from the HEK293- δ ag-S cells transfected with the myc epitope tagged mutant or wild type PSF, was quantified using spectrophotometry at 260nm. The RNA was run on a 1% native agarose gel to confirm its integrity. A PCR was also performed for β -Actin on the RNA sample to determine if genomic DNA contamination was present following the extraction. Once the integrity of the RNA sample was determined, 5 μ g of total RNA from each treatment was used to prepare cDNA using the iScript cDNA synthesis kit (Biorad) following the manufacturer's protocol. The random primer mix supplied by the manufacturer was used with a final concentration of (1nM) in a 20 μ L reaction mixture. The following conditions were used for cDNA synthesis: 5min at 25°C, 30min at 42°C and, 5 min at 85°C. The reaction was then kept at 4°C until the cDNA was ready for use in the QPCR reaction. Following the RT reaction, qPCR

was carried out using the IQ SYBR GREEN supermix (Biorad), where 5µL of total cDNA obtained from the RT reaction was used. Forward and reverse primers which synthesize the right terminal stem loop domain of HDV (R199G) were used with a final concentration of 1nM. The sequences for the forward (R199GFW-Figure 2.1) and reverse (R199GREV-Figure 2.1) primers are respectively: 5'-GGA ATT CTA ATA CGA CTC ACT ATA GGG ACT GCT CGA GGATC-3' and 5'-ACA TCC CCT CTC GGG TGC TG-3'. The sequences for the forward and reverse primers for the housekeeping gene β-Actin which synthesize the 5' end are respectively: 5'-ATG GAT GAT GAT ATC GCC GCG CTC GTC GTC-3'and 5'-GGG GTA CTT CAG GG TGA GGA TGC CTC TCTT-3' (Greco-Stewart thesis, 2009). The QPCR reactions were carried out using Chromo 4 (Biorad) with the Opticon 3 software (Biorad) in the following conditions: 95°C for 3 min, 95°C for 30s, 55°C for 30s and 72°C for 30 s for each cycle for a total of 40 cycles in a total reaction volume of 20µL. Primers were determined to be specific for both HDV (R199G) and β-Actin. Primer efficiency curves were performed for both HDV and β-Actin, and primer efficiencies were found to be within 10%. All experiments were done in triplicates. Technical replicates were performed for all biological replicates.

Following qPCR experiments a common THRESHOLD was picked and the corresponding average ct values obtained from the technical replicates were used to calculate the relative amount of HDV RNA and β-Actin mRNA present in the samples following the treatments. All samples were normalized against the mock treatment. All calculations were done manually in Microsoft excel 2007, using the following equation:

$$2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct \text{ reference}(\text{control-treated})] - [\Delta Ct \text{ target}(\text{control-treated})]}$$

Figure 2.1: Schematic representation of HDV RNA. The hepatitis *delta* genome is depicted here with the superimposition of both genomic and antigenomic polarities. The HDV RNA genome is shown here as a single stranded RNA molecule that folds to form an unbranched rod-like structure. The two complimentary ribozyme motifs are indicated. The locations of the forward and reverse primers used to generate R199G are indicated. The numbering is in accordance with Kuo *et al.*, 1988. (Figure adapted from Greco-Stewart *et al.*, 2007 Figure 1).



where *ct* is the cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold (Karlen *et al.*, 2007). Reference refers to β -Actin RNA, while target refers to HDV RNA, and control refers to the mock treatment.

Once the western blots for the myc tag and β -actin proteins were completed, the membranes were exposed for various times to different films in order to obtain a linear range for densitometry purposes. The density for the corresponding band was measured using Image J (Rasband, 2011). In order to determine the relative amount of protein in each sample, the intensity for the Myc tagged protein was standardized with the intensity of the β -Actin protein for the same sample, as a loading control. All protein samples were normalized against the myc-PSF protein.

The relative amount of cDNA for HDV following the qPCR experiment was standardized with the relative amount of β -Actin cDNA for the same treatment. All treatments were normalized against the cDNA obtained from the mock treatment. The mean and standard deviation were calculated for the three trials. An unpaired student t-test using Microsoft excel was calculated on all three trials of each treatment, which were compared against the mock treatment.

Because not all the transfections with the myc epitope tagged wild type and mutant PSF proteins resulted in the exact same relative amount of protein expression, I therefore standardized the cDNA with the normalized protein obtained for the same treatment. All treatments were normalized with the myc-PSF. The mean and standard deviation were calculated for the three trials. An unpaired student t-test using Microsoft excel was calculated on all three trials of each treatment, which were compared against the myc-PSF.

3.0 Results

3.1. *Ex-vivo* knockdown experiments of PSF confirmed that PSF is required for HDV RNA accumulation

In order to determine the physiological relevance of PSF in relation to HDV, I determined whether PSF was required for HDV RNA accumulation *ex-vivo*. Therefore, I used a commercial pool of three siRNAs designed to specifically knock down PSF expression. As for the controls, mock treatments and a pool of commercial five scrambled siRNAs were used. Both the controls and the siRNAs targeted against PSF mRNA were transfected in HEK293- δ ag-S cells carrying a mutant form of HDV RNA (Chang *et al.*, 2005a). The mutant form of HDV carries a two nucleotide deletion in a region that disrupts the expression of the δ ag-S, required for HDV RNA accumulation thus creating a frameshift mutation (Chang *et al.*, 2005a). The expression of the δ ag-S is provided by an external gene under the control of a TET promoter (Chang *et al.*, 2005a). However, the TET promoter is a 'leaky promoter' (Gossen and Bujard, 1992) that still expresses the δ ag-S at a low level in the absence of TET (Chang *et al.*, 2005a). Therefore, in the absence of TET, δ ag-S is still produced allowing for basal levels of HDV RNA to accumulate. But when the expression of the δ ag-S is induced, the δ ag-S is over produced which in turn over produces HDV RNA (Chang *et al.*, 2005a). Therefore, 24 hours post transfection, I induced the expression of the δ ag-S with 1 μ g/mL of tetracycline. 48 hours post transfection the cells were harvested and both the protein and RNA were extracted.

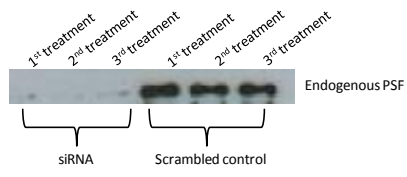
Following RT-qPCR experiments my results indicated that HDV RNA was overproduced by $\sim 34 \pm 7$ folds when the δ ag-S was induced, which is in agreement with ~ 40 folds to what has

been previously reported (Chang *et al.*, 2005 a). According to Chang and colleagues, this induction reaches a maximum of 4.3×10^4 HDV RNA copies per cell following 1 day of induction (Chang *et al.*, 2005a). Over the course of this treatment ~70% of the cells remained adherent indicating that these cells were slightly affected by the treatments. Western blot analysis for PSF (Figure 3.1 A) and the housekeeping protein β -Actin (Figure 3.1 B) were performed to confirm that the PSF knockdown was successful. Also, RT-qPCR experiments were performed to measure the relative amounts of HDV and β -Actin RNA that accumulated following the knockdown.

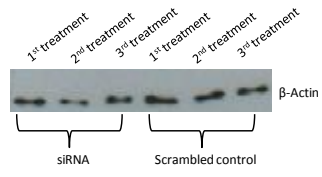
My results indicate that PSF was knocked down by >85% (Figure 3.1 C) when compared to the scrambled control. The siRNA knockdown resulted in significantly lower HDV RNA accumulation by 40 % (Figure 3.1 D) when compared to the scrambled control. Therefore, because HDV RNA accumulation was significantly lowered following the knockdown of PSF my results suggest that PSF could be directly or indirectly involved in HDV replication.

Figure 3.1: The knockdown of PSF in HDV replicating cells over producing HDV RNA. The physiological relevance of PSF in relation to HDV was determined by using siRNAs designed to specifically knock down PSF expression. As for the controls, mock treatments and scrambled siRNAs were used. Both the controls and the siRNAs targeted against PSF mRNA were transfected in HEK293- δ ag-S cells carrying a mutant form of HDV RNA. The PSF knockdown is confirmed here through western studies detecting PSF A) and β -Actin B). C) The relative amount of PSF was quantified in each treatment relative to the housekeeping β -Actin protein. D) HDV RNA accumulation was measured in each treatment following the transfection with the scrambled controls and the siRNA's directed against PSF gene expression. The relative amount of HDV RNA was normalized with the relative amount of β -Actin RNA through RT-qPCR experiments. Experiments were performed n=3 to obtain a standard deviation. P-values (student t-test unpaired distribution) were obtained and are indicated above the bars. Asterticks indicate significant difference $p < 0.05$.

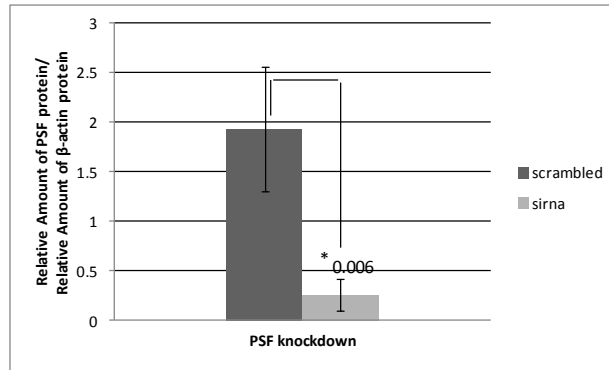
A)



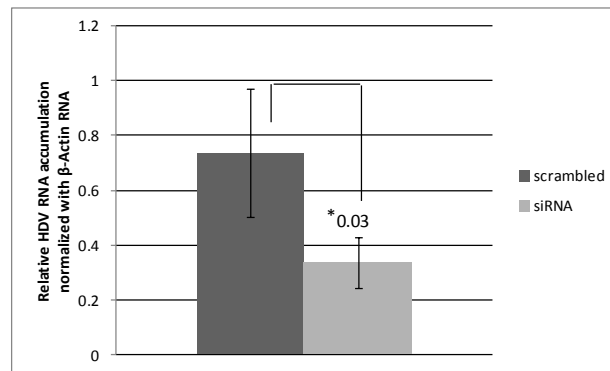
B)



C)



D)



3.2. *Ex-vivo* mutagenesis experiments identified the PSF domains required for basal levels of HDV RNA accumulation

The previous experiments linked PSF to HDV RNA accumulation. These experiments demonstrated that following the knockdown of PSF in HDV replicating cells, HDV RNA accumulation was significantly lower in comparison to the scrambled controls. Furthermore previous experiments demonstrated that PSF-HDV interaction was required for HDV to interact with RNAPII, and both PSF and RNAPII were found to simultaneously interact with HDV RNA (Greco-Stewart Thesis, 2009). To further investigate the role of PSF in relation to HDV RNA accumulation, I investigated which PSF domains were required for HDV RNA accumulation *ex-vivo*.

I utilized a library of N-terminal c-myc-epitope tagged wild type and mutants PSF proteins that were constructed by deleting each PSF domain (Figure 3.2 A) (Rosonina *et al.*, 2005). The N-terminal domain of PSF has been previously shown to be essential for binding to the CTD of RNAPII (Rosonina *et al.*, 2005). Thus, the N-terminal domain might be essential for protein-protein interactions required for HDV RNA accumulation. While the RNA recognition motifs (RRM1 and RRM2) might be essential for binding to HDV RNA for HDV RNA accumulation.

Each of the PSF constructs were first transfected in HEK293- δ ag-S cells. I investigated the effect of these mutants on residual basal levels of HDV RNA accumulation. According to Chang and colleagues the HEK293- δ ag-S cells produce ~1000 copies (basal levels) of mutated HDV RNA per cell when not induced (Chang *et al.*, 2005a). 48 hours post transfection the cells were harvested and both protein and RNA were extracted. Over the

course of this treatment ~90% of the cells remained adherent indicating that these cells were not greatly affected by the treatments.

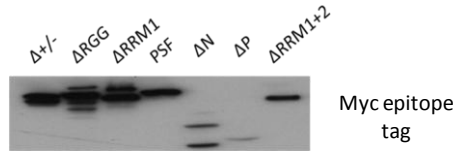
Following the exposure of the western blot membrane onto an x-ray film, the density of the protein band was measured using Image J. The density of the band corresponding to the myc tagged protein (Figure 3.2 B) was normalized with the density for the band corresponding to the β -Actin protein (Figure 3.2 C) for the same treatment as a loading control. I also performed western blot analysis for endogenous PSF (data not shown) to determine the relative amount of myc epitope tagged proteins that were expressed in comparison to endogenous PSF. The expression levels of all myc-PSF and mutant PSF proteins are relatively similar to each other and are expressed at similar levels as endogenous PSF, with the exception of the myc- Δ N and myc- Δ P mutants (Figure 3.2 C). This is inconsistent to what has been previously reported by Rosonina and colleagues, where all myc-PSF and the myc mutant PSF proteins were expressed similarly to each other and to endogenous PSF (Rosonina *et al.*, 2005). This might be because I utilized a different cell system from the Blencowe laboratory which utilized unmodified HEK293 cells, and perhaps the Δ N and Δ P mutants stressed the HEK293- δ ag-S cells, resulting in low protein expression for each mutant. This hypothesis is consistent to the two bands observed with Δ N similar to what has been previously reported, which might suggest proteolysis (Rosonina *et al.*, 2005). In order to measure the relative amounts of HDV and β -Actin RNA, RT-qPCR experiments were performed for HDV and β -Actin RNA. The relative amount of HDV RNA was standardized with the relative amount of β -Actin RNA for the same treatment. All treatments were normalized with the mock treatment (Figure 3.2 D).

Figure 3.2: *Ex-vivo* experiments of PSF mutants transfected in HDV replicating cells expressing basal levels of HDV RNA. A) Depicted here is a schematic representation of PSF. The ΔN mutant is a deletion of the P,P/Q and RGG domains. The $\Delta RRM1+2$ mutant is a deletion of the RRM1 and RRM2 domains. Myc-epitope tagged *wt* and PSF mutants were transfected in HDV replicating cells expressing basal levels of HDV RNA accumulation. B) Myc tagged protein obtained through western studies using α -myc antibody. C) β -Actin protein obtained through western studies using α - β -Actin antibody. The myc tagged protein was standardized with β -Actin protein. D) QPCR experiments were done to quantify the effects the PSF mutants had on basal levels of HDV RNA accumulation. Experiments were standardized with the mock treatment. E) QPCR experiments were normalized with the relative amount of protein for the same treatment. All results were standardized against myc-PSF. The PSF mutants that significantly effected HDV RNA accumulation are $\Delta RRM1$, ΔP and $\Delta RRM1+2$ indicated with *. Experiments were performed n=3 to obtain a standard deviation. P-values (student t-test unpaired distribution) were obtained indicated above the bars. Asterticks indicate significant difference $p < 0.05$.

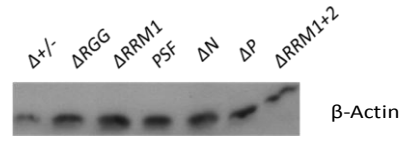
A)



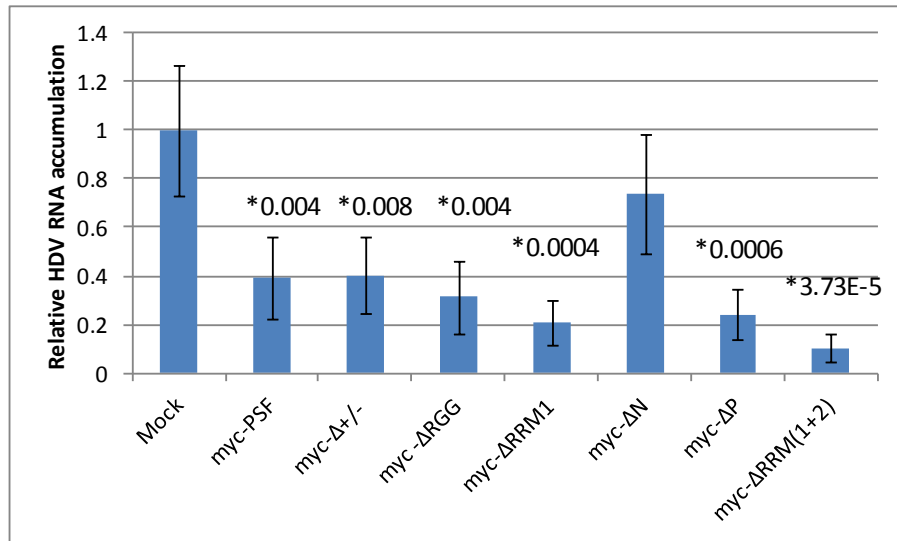
B)



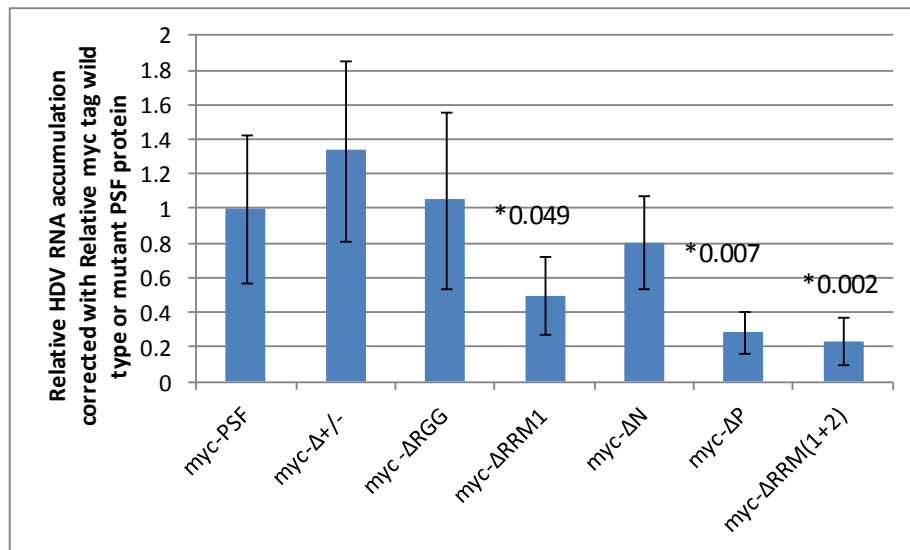
C)



D)



E)



Based on my results (Figure 3.2 D) the transfections with the myc-PSF, myc- Δ +/-, myc- Δ RGG, myc- Δ RRM1, myc- Δ P and myc- Δ RRM1+2 mutants resulted in significantly lower HDV RNA accumulation, in comparison to the mock treatment. This makes all the myc wild type and myc PSF mutants significantly different from the mock, with the exception of the myc- Δ N mutant. This data also indicates that the myc wild type and PSF mutants have an inhibitory effect on HDV RNA accumulation, indicating that the myc tag located at the N-terminal domain of all the proteins might be affecting their proper folding, thus indirectly affecting HDV RNA accumulation.

Because not all the transfections with the myc epitope tagged wild type and mutant PSF proteins resulted in the same relative amount of protein expression, I therefore standardized the relative amount of HDV RNA for the normalized protein obtained for the same treatment (Figure 3.2 E). All treatments were normalized with the myc-PSF.

Based on my results, the, myc- Δ RRM1, myc- Δ P and myc- Δ RRM(1+2) resulted in significantly lower HDV RNA accumulation, in comparison to the myc-PSF. Because the myc- Δ P, myc- Δ RRM1 and myc- Δ RRM(1+2) mutants significantly decreased HDV RNA accumulation in comparison to the myc-PSF (Figure 3.2 D and E) then the domains that might be required for basal levels of HDV RNA accumulation are the P, RRM1 and RRM2 domains. However, it should be noted that the Δ RRM2 mutant should also be tested to see whether it affects HDV RNA accumulation. Therefore, we cannot conclude from this data alone that the RRM2 domain is required for HDV RNA accumulation.

3.3. *Ex-vivo* mutagenesis experiments to determine which PSF domains might be required for the over-production of HDV RNA accumulation

Secondly, I investigated the effect of these mutants following the induction of HDV RNA accumulation. To complement my previous experiment, the purpose of this study was to allow me to distinguish between residual HDV RNA accumulation as compared to elevated HDV replication. The HEK293- δ ag-S cells were transfected as described above, but this time I induced the expression of the δ ag-S using 1 μ g/mL of TET. 48 hours post transfection the cells were harvested and both protein and RNA were extracted. Western blot analysis for β -Actin and the Myc tagged wild type and mutant PSF proteins were performed. Over the course of this treatment ~55% of the cells remained adherent indicating that these cells were affected by the treatments. This is consistent with the Taylor lab which reported that 2 days following the induction, there was a fivefold reduction of adherent cells compared to uninduced cells (Chang *et al.*, 2005a) and by day 3 there was a 60% decrease in total cell number in induced cells (Chang *et al.*, 2005a). They found that HDV RNA replication was interfering with cell growth, cell cycle, cell death and cell adherence (Chang *et al.*, 2005a). Thus, HDV genome replication was indirectly cytotoxic (Chang *et al.*, 2005a). However, it is worth noting that I also used the same method of induction for results (3.1) and only 30% of the cells lifted. The reason why the cells responded differently in each case could be because the two experiments were not done simultaneously. Rather the knockdown experiment (3.1) was performed a few months prior to experiment (3.3).

Following critical analyses of the raw data, the experiment was deemed inconclusive due to the fact that the biological replicates were not reproducible (data not shown). This might be

attributed to the fact that the cells were greatly affected by the induction with tetracycline which could have caused a different response from one biological replicate to the next.

3.4. *In vitro* co-immunoprecipitation experiments were performed to detect interactions between RNAPII and the PSF mutants

My results demonstrate that PSF is required for HDV RNA accumulation and the deletion of certain PSF domains might affect this accumulation. In order to further investigate my hypothesis that '*PSF acts as a transcription factor during HDV RNA replication by interacting with both the CTD of RNAPII and HDV RNA simultaneously*', I then aimed to determine which PSF domains were interacting with RNAPII. As described above, I transfected HEK293 cells with myc-epitope tagged wild type and PSF mutants. 48 hours post transfection I prepared the protein extracts and subjected 50µg of protein extracts to the co-immunoprecipitation assay, as previously performed (Greco-Stewart *et al.*, 2006). 50µg of protein extract was found to be sufficient to co-immunoprecipitate the protein complexes. I waited 48 hours post transfection to harvest the cells since all my experiments were performed in a 48 hour time period. In order to determine whether protein-protein interactions between wild type or mutant PSF proteins and RNAPII are independent of endogenous RNA and DNA, I pre-incubated the protein extract with RNase and DNase prior to the co-immunoprecipitation experiment. I utilized an α-IgG as a control to test for non-specific interactions between the protein complexes, the beads and the antibody itself (Abraham and Pelchat, 2008).

First I utilized the α-myc tag antibody to co-immunoprecipitate the myc tagged PSF mutants. Following the co-immunoprecipitation assay, a western blot was performed for

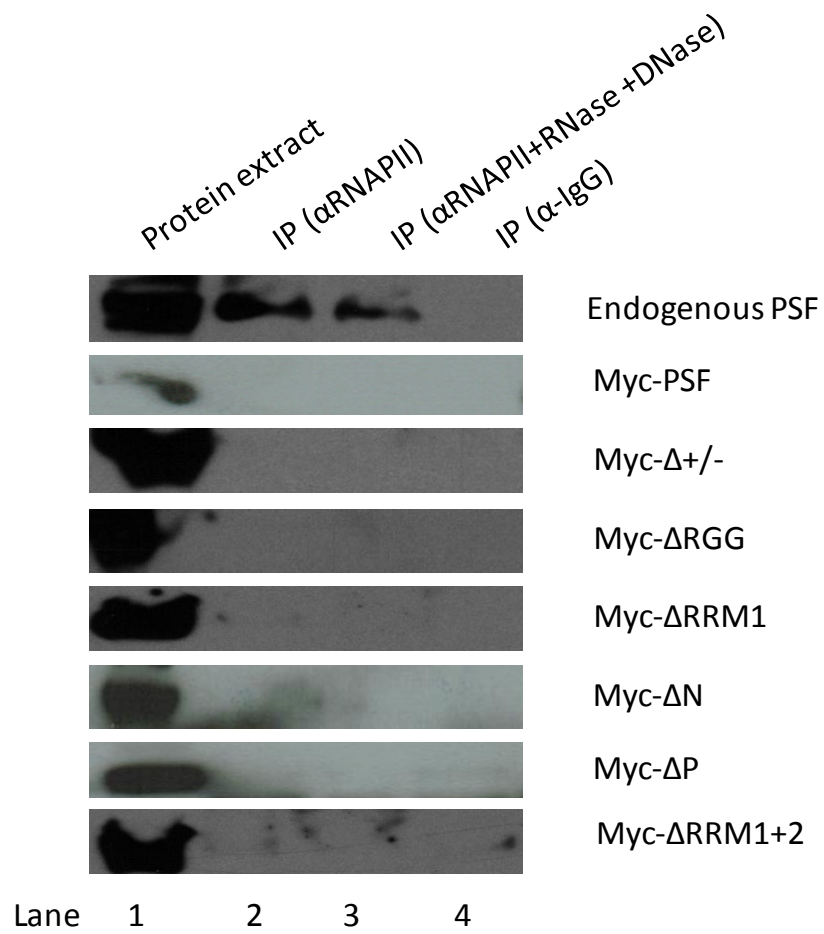
the myc tag, to detect if the myc epitope tagged wild type and PSF mutants immunoprecipitated. No protein was detected following the western blot analysis (data not shown) despite trying several times and several different conditions. Therefore, it was evident that the co-immunoprecipitation experiment using the α -myc tag antibody did not co-immunoprecipitate the myc tagged wild type and mutant PSF proteins. This might be because the N-terminal C-myc tag was embedded within the tertiary structure of PSF making it inaccessible by the α -myc antibody.

Thus, I performed the reciprocal experiment, but using an α -RNAPII antibody (8WG16) raised against the CTD of RNAPII. This was followed by a western blot analysis for endogenous PSF (Figure 3.3) to detect if endogenous PSF was able to co-immunoprecipitate with RNAPII, and indeed it did. Although not quantitative, this interaction between endogenous PSF and RNAPII seems to diminish when the protein extract was pre-treated with RNase and DNase. Furthermore, the interaction between PSF and RNAPII is specific to α -RNAPII, because only the co-immunoprecipitation with α -RNAPII was able to immunoprecipitate PSF, while the α -IgG antibody was unable to co-immunoprecipitate PSF. Also as previously reported (Greco-Stewart thesis, 2009) a co-immunoprecipitation using α -PSF antibody can also co-immunoprecipitate RNAPII.

Because I was able to co-immunoprecipitate PSF utilizing α -RNAPII, I therefore utilized α -RNAPII for my co-immunoprecipitation experiments to determine if any of the wild type or mutant PSF proteins were able to co-immunoprecipitate with RNAPII.

Based on my results (Figure 3.3-lane 1) the myc-proteins were all expressed in the protein extracts. However, it is apparent that neither the myc wild type nor the mutant PSF

Figure 3.3: Interaction of RNAPII with PSF mutants. Co-immunoprecipitation assays using α -RNAPII were performed on protein extracts obtained from HEK293 cells transfected with myc-epitope tagged mutant and wild type PSF proteins. A western blot for endogenous PSF was done to detect if endogenous PSF was co-immunoprecipitating with RNAPII. Controls included pre-incubating the protein extracts with RNase and DNase to determine if protein-protein interactions are independent of DNA or RNA. The co-immunoprecipitation with α -IgG was used to determine the specificity of the interaction. Following the co-immunoprecipitations, the eluted proteins were subjected to an SDS-PAGE followed by a western to detect the myc tag.



proteins were able to co-immunoprecipitate with RNAPII indicating that none of the myc wild type nor the mutant PSF proteins interact with RNAPII. Because the α -myc antibody was unable to immunoprecipitate the myc tagged proteins, while the myc-PSF was unable to co-immunoprecipitate with RNAPII, and all the myc-PSF and mutant proteins were inhibiting HDV RNA accumulation in comparison to the mock control (Figure 3.2 D), this might suggest that all the myc wild type and mutant PSF proteins were being affected by the myc epitope tag located at the N-terminal domain. The myc tag could be interfering with the proper folding of the proteins thus affecting proper protein function.

A summary of the results for the knockdown of PSF, the transfections with the myc wild type and mutant PSF proteins, and the co-immunoprecipitation experiments between the PSF mutants and RNAPII are presented in Table 3.1.

Table 3.1: Summary of results for endogenous PSF and the myc-wild type and mutant PSF proteins. The *in vitro* interactions between RNAPII and the various mutant myc-PSF proteins, the *ex-vivo* assay for the PSF knockdown and its effect on HDV RNA accumulation. The *ex-vivo* assay for the PSF mutants and their effect on HDV RNA accumulation in comparison to the mock and the myc-PSF treatments.

PSF domain	<i>In vitro</i> interaction with RNAPII	Effect on relative HDV RNA accumulation in comparison to the mock	Effect on relative HDV RNA accumulation in comparison to the myc-PSF
Endogenous PSF	<i>Interacted with RNAPII</i>	<i>Knockdown of PSF decreases relative HDV RNA Accumulation</i>	<i>N/A</i>
Myc- PSF	<i>Did not interact with RNAPII</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>	<i>N/A</i>
Myc-Δ+/-	<i>Did not interact with RNAPII</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>	<i>Slight increase in basal levels of relative HDV RNA accumulation</i>
Myc-ΔRGG	<i>Did not interact with RNAPII</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>	<i>No change in basal levels of relative HDV RNA accumulation</i>
Myc-ΔRRM1	<i>Did not interact with RNAPII</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>
Myc-ΔN	<i>Did not interact with RNAPII</i>	<i>Lowered basal levels of relative HDV RNA Accumulation</i>	<i>Lowered basal levels of relative HDV RNA Accumulation</i>
Myc-ΔP	<i>Did not interact with RNAPII</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>
Myc-ΔRRM1+2	<i>Did not interact with RNAPII</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>	<i>Significantly Lowered basal levels of relative HDV RNA Accumulation</i>

4.0. Discussion

4.1. Interaction of HDV with the host RNAPII and PSF

HDV possesses limited protein coding capacity and thus relies on host proteins for transcription and replication. Identifying and characterizing the host proteins that HDV relies on during its life cycle will further characterize the transcriptional machinery required for HDV RNA transcription and replication. This would provide a better understanding of how RNAPII selects its RNA promoter to transcribe from an RNA template.

My thesis determined the physiological relevance of PSF in relation to HDV RNA accumulation *ex-vivo*. To understand the relationship of PSF to HDV RNA, I knocked down PSF in HDV replicating cells, and determined whether this knockdown had an adverse effect on HDV RNA accumulation. In order to test whether the PSF knockdown affected the over production of HDV RNA accumulation, I induced the expression of the δ ag-S in the HEK293- δ ag-S cell line (Chang *et al.*, 2005a). Based on my results, the PSF knockdown resulted in significantly lower HDV RNA accumulation. Furthermore, previous experiments reported that HDV RNA had to interact with PSF in order for HDV RNA to interact with RNAPII (Greco-Stewart Thesis, 2009), and both PSF and RNAPII were found to simultaneously interact with HDV RNA (Greco-Stewart Thesis, 2009). Together, the above studies suggest that the physiological relevance of PSF in relation to HDV RNA might be to act as a transcription factor during HDV RNA replication.

The fact that PSF can act as a transcription factor has been previously reported by various studies. A study reported that PSF in a complex with p54^{nrb} (Dong *et al.*, 1993), which has been previously reported to be an HDV interacting protein (Sikora *et al.*, 2009), can co-

regulate the transcriptional activity of androgen receptors in order to control the expression of the transcription products (Dong *et al.*, 2007). Rosonina and colleagues reported that PSF and p54^{nrb} are recruited to activated promoters and the CTD of RNAPII to mediate the co-transcription of pre-mRNA processing *in vivo* (Rosonina *et al.*, 2005). These studies could suggest that the complex formed between PSF and p54^{nrb} might have been inhibited when PSF was knocked down in my experiments. The complex between PSF and p54^{nrb} might have been required for HDV RNA transcription, thus affecting HDV RNA accumulation. However, it has not been determined to date whether the interaction between PSF to p54^{nrb} is essential for HDV RNA accumulation. Furthermore, we cannot ignore the fact that PSF is an RNA processing protein which has been referred to as a 'sticky' protein since it has the ability to dimerize with p54^{nrb} or remain as a monomer, and bind RNA/DNA, or interact with other nuclear factors (Shav-Tal and Zipori, 2002). Therefore, PSF might also be involved in other nuclear processes other than HDV RNA accumulation, and when PSF was knocked down, these nuclear processes might have been inhibited, which could have indirectly affected HDV RNA accumulation.

A study has reported that the PSF/p54^{nrb} complex is associated with the nuclear retention, and binding to defective RNAs, to allow for the subsequent editing of hyperedited inosine-rich RNAs (Zhang and Carmichael, 2001). Another study reported that PSF regulated post transcriptional mRNA stability of the human immunodeficiency virus type 1 (HIV-1) (Zolotukhin *et al.*, 2003). Both studies suggest that there might be a preference for PSF to bind to certain types of RNAs in order to localize the RNAs to the nucleus (Zhang and Carmichael, 2001; Zolotukhin *et al.*, 2003). Therefore, when PSF was knocked down in my

experiments, PSF might have lost its interaction with HDV RNA, and this in turn might have prevented HDV RNA from being localized to the nucleus, which in turn could have caused the low levels of HDV RNA accumulation. However, this has not been tested.

Although PSF was knocked down by more than 85%, I observed only 40% reduction of HDV RNA accumulation. Since PSF is a multi-functional protein (Patton *et al.*, 1993) the knockdown of PSF might pose an inconvenience for the cells, which might have produced more non-transfected cells, which could be why some residual HDV RNA still remains following the knockdown. The accumulation of residual HDV RNA can also be attributed to HDV RNA stability. Unit length HDV RNA has been previously reported to be very stable and resistant to dicer endonuclease activity (Chang *et al.*, 2003). Dicer cleaves RNA substrates that are 100% double stranded into double stranded RNA fragments which are ~21 nt referred to as small interfering RNAs (siRNAs) (Hammond *et al.*, 2000; Chang *et al.*, 2003). These siRNAs can be incorporated into an RNA-induced silencing complex (RISC) in order to produce endonuclease activity that cleaves related mRNA species. Dicer can also cleave RNAs that form intramolecular base pairing with <100% base pairs (Hammond *et al.*, 2000; Chang *et al.*, 2003). Thus, Chang and colleagues investigated whether HDV RNA accumulation can be affected by dicer activity, since HDV RNA can fold into a rod-like structure with 74% intramolecular base pairing (Chang *et al.*, 2003). According to Chang and colleagues, HDV RNA accumulation was not affected by dicer, because HDV RNA fragments of ~21nt were undetectable following an HDV RNA transfection (Chang *et al.*, 2003). Thus, they concluded that unit length HDV genomic and antigenomic RNAs are extremely stable RNA molecules, which are resistant to dicer activity. (Chang *et al.*, 2003). According to my

results, the reason why residual RNA remained following the knockdown of PSF could also be because HDV RNA is a 'stable' RNA molecule that remained intact, even though PSF was present in limiting amounts.

We cannot ignore the fact that the δ ag-S required for HDV RNA replication (Gudima *et al.*, 1999) was present in high concentrations following the knockdown of PSF. This is because the expression of the δ ag-S was induced, therefore low levels of HDV RNA could have accumulated simply due to the abundance of the δ ag-S. Furthermore, a low amount of endogenous PSF remained following the knockdown, and so low levels of HDV RNA could have accumulated from intact endogenous PSF.

In this cell system, the induction of the δ ag-S overproduced HDV RNA by a relative amount of $\sim 34 \pm 7$ folds, compared to ~ 40 folds to what has been previously reported (Chang *et al.*, 2005a). The small experimental difference could have arisen if different concentrations of antibiotics were utilized for the maintenance of the HEK293- δ ag-S cell line (Chang *et al.*, 2005a). This would have been done in order to minimize stress induced by antibiotics on cells, which would in turn cause a difference in the overproduction of HDV RNA. However, in my knockdown experiment, only $\sim 70\%$ of the cells remained adherent following the induction for 24 hours. Therefore, I assume that if I left the induction for a longer period of time, more cell death could have resulted. As previously reported by the Taylor lab, HDV genome replication was found to be cytotoxic (Chang *et al.*, 2005a). Nevertheless, this system remains the only system that can achieve HDV replication by simply adding TET instead of utilizing disruptive procedures such as electroporation of DNA or RNA.

Now that I have determined the physiological relevance of PSF in relation to HDV RNA accumulation *ex-vivo*, I wanted to further investigate this relationship. Therefore, I asked which PSF domains are required for HDV RNA accumulation *ex-vivo*, and if these PSF domains are required to interact with RNAPII *in vitro*. Together these experiments further investigated the hypothesis that '*PSF acts as a transcription factor during HDV RNA replication by interacting with both the CTD of RNAPII and HDV RNA simultaneously*'.

The *ex-vivo* experiments were designed to determine which PSF domains affected HDV RNA accumulation. In order to determine which PSF domains affected basal levels of HDV RNA accumulation, I utilized the HEK293- δ ag-S cell model (Chang *et al.*, 2005a), but I did not induce the expression of the δ ag-S following transfection of the myc epitope tagged PSF proteins. As for the *in vitro* experiments they were designed to determine which PSF domains were required to bind to RNAPII. The *in vitro* co-immunoprecipitation assays were performed by utilizing protein extracts prepared from HEK293 cells transfected with the myc-epitope tagged PSF proteins (Rosonina *et al.*, 2005), along with an antibody raised against the CTD of RNAPII. The co-immunoprecipitation experiments were followed by western blot analyses to detect the myc epitope tagged proteins. In order to determine whether protein-protein interactions between the myc epitope tagged PSF proteins and RNAPII are independent of RNA and/or DNA, I pre-incubated the protein extract with RNase and DNase. I replaced the α -RNAPII antibody with α -IgG as a control for non-specific interactions (Abraham and Pelchat, 2008).

According to my co-immunoprecipitation experiments, the α -myc antibody was unable to immunoprecipitate the myc-PSF. Also, the N-terminal tagged protein myc-PSF did not co-

immunoprecipitate with RNAPII, despite a clear association of the endogenous PSF with RNAP II. Furthermore, all the mutants were unable to interact with RNAPII, in all conditions tested. Finally, *ex-vivo* experiments clearly demonstrated that all the myc-PSF and mutant PSF proteins resulted in lower HDV RNA accumulation in comparison to the mock control. Taken together, a possible explanation as to why the wild type myc-PSF was unable to interact with RNAPII and was not recognized by the α -myc antibody, is that the myc tag located at the N-terminal part of the protein affected the proper folding of this protein segment thus its proper function. As previously reported (Rosonina *et al.*, 2005) the N-terminal domain of PSF seems to be required to interact with the CTD of RNAPII. Taken together this might suggest why the myc tag located at the N-terminal domain of PSF might have prevented myc-PSF from interacting with RNAPII thus lowering HDV RNA accumulation in comparison to the mock control. However, it should be noted that although none of the mutants and the wild type myc tagged PSF proteins interacted with RNAPII *in vitro*, they could have slightly interacted with RNAPII or other essential proteins required for HDV RNA accumulation *ex-vivo*, thus producing low levels of HDV RNA accumulation in comparison to the mock control. On the other hand the fact that the myc wild type and mutant PSF proteins lowered HDV RNA accumulation in comparison to the mock control and did not produce an interaction with RNAPII *in vitro*, might further suggest that these proteins could be acting as dominant negative inhibitors.

Although the myc tag might have affected the proper folding and function of the myc tagged proteins, the ΔP , $\Delta RRM1$, and the $\Delta RRM1+2$ mutants were still able to significantly reduce HDV RNA accumulation in comparison to the wild type myc tag PSF. According to

the *in vitro* experiments performed by the Blencowe laboratory, the Δ RRM1 mutant was able to produce an interaction with the CTD of RNAPII, while the Δ P and Δ RRM1+2 mutants did not produce an interaction with the CTD of RNAPII (Rosonina *et al.*, 2005). A possible explanation as to why the *in vitro* experiments performed by the Blencowe laboratory yielded different outcomes from my *in vitro* experiments could be because a different experimental approach was taken by the Blencowe laboratory. The Blencowe laboratory performed GST pull down experiments which utilized protein extracts prepared from HEK293 cells transfected with the myc-epitope tagged PSF mutants (Rosonina *et al.*, 2005). The protein extracts were incubated with GST fusion proteins containing a wild type RNAPII CTD peptide (Rosonina *et al.*, 2005). The protein extracts were then subjected onto glutathione sepharose beads that were washed extensively, and bound proteins were eluted, and analyzed by immunoblotting to detect the myc tagged PSF mutants (Rosonina *et al.*, 2005). Thus, this experiment is different from my *in vitro* experiment since I utilized the complete RNAPII protein for co-immunoprecipitation, and not just the CTD peptide of RNAPII.

Asforementioned, my *ex-vivo* experiments demonstrated that both the Δ RRM1 and Δ RRM1+2 mutants resulted in significantly lower HDV RNA accumulation. Also, recently in our laboratory Dajiang Zhang found that both the Δ RRM1 and Δ RRM1+2 mutants did not interact with HDV RNA (Dajiang Zhang, *unpublished data*). Taken together, these results could indicate that both the RRM1 and RRM2 domains might be required to bind HDV RNA for HDV RNA accumulation. However, it should be noted that the Δ RRM2 mutant should

also be tested to see whether it does not interact with HDV RNA and if it affects HDV RNA accumulation.

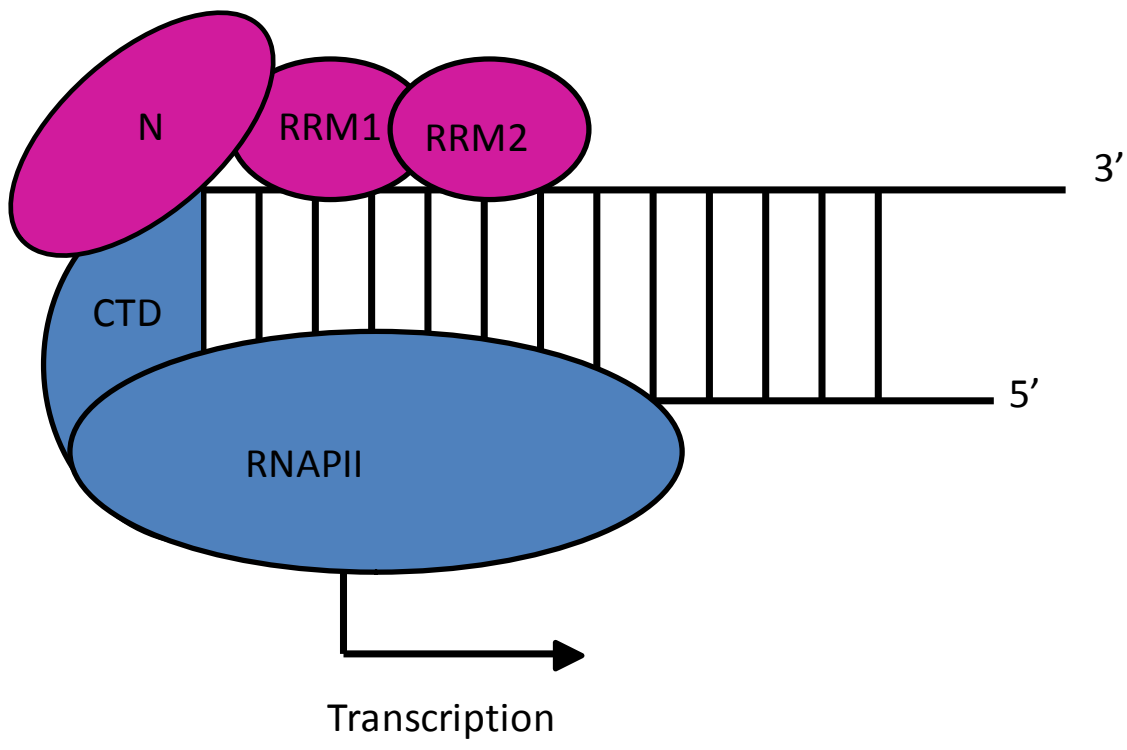
Another good experiment to complement my *in vitro* and *ex-vivo* experiments is to determine whether the myc tagged PSF proteins interact with HDV RNA utilizing RNA chromatography experiments. To further determine whether PSF might act as a transcription factor during HDV RNA replication, an *in vitro* transcription assay utilizing a nuclear extract with immunodepleted PSF should be performed.

5.0 Concluding remarks

My results suggest that HDV RNA accumulation requires PSF, and the PSF domains that might be required for HDV RNA accumulation are the N-terminal domain and the C-terminal RNA recognition motifs RRM1 and RRM2 (Figure 5.1).

Even though the above experiments suggest that PSF is required for HDV RNA accumulation, we cannot ignore the fact that PSF is involved in many nuclear processes which might be inhibited either in the absence of PSF and/or in the presence of the myc wild type and mutant PSF proteins. My thesis, in addition to the proposed studies provide a better understanding of how an RNA promoter might be recognized by RNAPII. Nevertheless, the elucidations of other host factors are yet to be determined to enhance our comprehension in the pathogenesis of subviral RNA replication.

Figure 5.1: Schematic representation of my evolved model. PSF might act as a transcription factor during HDV RNA accumulation through the C-terminal domains RRM1 and RRM2 which might interact with HDV RNA, while the N-terminal domain might interact with the Carboxy terminal domain (CTD) of RNAPII.



6.0. References

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7.0. Contribution of Collaborators

All of the laboratory experiments were performed by Youser Al-Ali

8.0. Curriculum Vitae

Youser Al-Ali

Education

- 2008-2011 M.Sc. (Biochemistry), University of Ottawa, Ottawa
Thesis title: Investigation of the polypyrimidine tract-binding protein-associated splicing factor (PSF) domains required for the hepatitis *delta* virus (HDV) replication
- 2004-2007 B.Sc. (Biochemistry/Biotechnology), Carleton University, Ottawa
- 2000-2003 High school diploma, St. Paul Catholic High school, Ottawa

Work Experience

- 2009-2010 Senior Teaching assistant/Laboratory demonstrator for second year undergraduate biochemistry laboratories. Faculty of Science, University of Ottawa
- 2009-2010 Teaching assistant/Laboratory demonstrator for third year undergraduate biochemistry laboratories. Faculty of Science, University of Ottawa
- 2009-2010 Marker/Proctor for the undergraduate Biochemistry course (Metabolism)
- 2007-2008 Research Technician:
- Optimized an *in vitro* transfection assay for the study of the polypyrimidine tract binding protein associated splicing factor (PSF) in relation to HDV RNA accumulation
 - Trained other students with various biochemistry/Molecular biology techniques
 - Assisted other students with their research

- Conducted orders, prepared media, reagents and ensured the lab was always clean and organized
- Monitored radioactive levels weekly
- Dealt with business professionals from various scientific backgrounds
- Dealt with administration enquiries

Professional activity

- 2010 **Al-Ali, Youser, Greco-Stewart, Valerie and Pelchat, Martin.**
 Replication of the Hepatitis *delta* virus requires the binding of the both the RNA polymerase II (RNAPII) and the Polypyrimidine tract-binding protein associated splicing factor(PSF) proteins. (Riboclub) Opening Session, Universite de Sherbrooke, Quebec.
- 2010 **Al-Ali, Youser, Greco-Stewart, Valerie and Pelchat, Martin.**
 Replication of the Hepatitis *delta* virus requires the binding of the both the RNA polymerase II (RNAPII) and the Polypyrimidine tract-binding protein associated splicing factor(PSF) proteins. Biochemistry Microbiology and Immunology Annual Graduate Seminars, University of Ottawa.
- 2009 **Al-Ali, Youser, Greco-Stewart, Valerie and Pelchat, Martin.**
 Replication of the Hepatitis *delta* virus requires the binding of the both the RNA polymerase II (RNAPII) and the Polypyrimidine tract-binding protein associated splicing factor(PSF) proteins. Biochemistry Microbiology and Immunology Annual Graduate Poster Sessions, University of Ottawa.
- 2008 **Al-Ali, Youser, Greco-Stewart, Valerie and Pelchat, Martin.**
 Replication of the Hepatitis *delta* virus requires the binding of the both the RNA polymerase II (RNAPII) and the Polypyrimidine tract-binding protein associated splicing factor(PSF) proteins. (Riboclub) Opening Session, Universite de Sherbrooke, Quebec

Laboratory skills

Microbiology Preparation of Cell Culture media. Excellent *ex-vivo* Transfection skills. Bacteriology. Using bacterial growth assays to isolate species of interest including DNA and protein purification. Light fluorescence

Molecular Biology DNA, and RNA isolation, Cloning, Protein Purification, Plasmid Preparation, Radioactive labelling (^{32}P), RNA analysis and quantification (RT-QPCR), PCR, Western blots, Northern Blots, Agarose gels, Polyacrylamide gels, SDS-PAGE, Electrophoretic mobility shift assay (EMSA), immunoprecipitation assays and Enzyme kinetics

Analytical IR, ^1H NMR, ^{13}C NMR, Mass spectrometry. Microplate reader, Spectrofluorometer, Chromatography (TLC, HPLC)

Computer skills Programs in Windows operating systems, Microsoft word, Powerpoint, Excel, Wordperfect, Photoshop, Corel Draw, Internet Explorer, bioinformatics (NCBI databases, BLAST, sequence analysis, mFold)

Communication skills

Excellent leadership, teamwork and communication skills

Languages English: Advanced reading, written and oral skills

French: Intermediate reading, basic written & oral skills

Arabic: Advanced reading, written and oral skills

References

Available upon request